STRATEGIES TO INCREASE HIGHLY-UNSATURATED N-3 FATTY ACIDS IN RAINBOW TROUT FED VEGETABLE OILS

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By

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ABSTRACT

A series of experiments were conducted to examine the effect of petroselinic acid, found in coriander oil, on fillet, hepatocyte and whole body FA composition and Δ6 desaturase gene expression in hepatocytes of rainbow trout (*Oncorhynchus mykiss*) fed vegetable oil (VO) based diets containing no fishmeal (FM) or fish oil (FO). In the first experiment, rainbow trout were fed one of eight diets containing fish, flax, canola or *Camelina sativa* oil with or without coriander oil. Coriander oil in the diet increased concentrations of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3, $P < 0.05$) in the fillet. There was a trend to increased 20:5n-3 + 22:6n-3/20:4n-6 ratios when coriander oil was added to the diet ($P = 0.067$). The second trial set out to investigate the effects of varied levels of coriander oil in canola oil based diets, on i) the capacity of rainbow trout hepatocytes to desaturate, elongate and esterify [1-^{14}C] α-linolenic acid (18:3n-3; ALA) and [1-^{14}C] linoleic acid (18:2n-6, LA), ii) reducing the production of 20:4n-6 in hepatocytes and iii) gene expression. This experiment demonstrated a significant increase in 22:6n-3 ($P = 0.011$) and a decrease 20:4n-6 ($P = 0.023$) in rainbow trout hepatocytes. Furthermore, there was a three-fold decrease in acetate for the [1-^{14}C] 18:2n-6 and nearly a two-fold increase for the [1-^{14}C] 18:3n-3 substrate when coriander was added at increasing levels, illustrating an increase in peroxisomal β-oxidation. Relative gene expression of Δ6 desaturase decreased with addition of coriander oil at the 0.5 inclusion level of coriander oil. The final experiment was conducted to determine if bypassing the first Δ6 desaturase and rate-limiting step in the n-3 FA pathway causes increased conversion of stearidonic acid (SDA; 18:4n-3) to 20:5n-3 and 22:6n-3. Rainbow trout were fed one of six diets containing either fish, conventional linseed (flax), or SDA enriched linseed oil (LO) with and without coriander oil. Inclusion of coriander oil did not affect any growth or feed intake parameters of rainbow trout.

However, the addition of coriander oil caused a significant increase in whole body 22:6n-3 and 20:4n-6 concentrations in fish fed SDA enriched LO with coriander oil (SDA^{+C}) compared to fish fed conventional linseed oil (LO). These results suggest that petroselinic acid, found in coriander oil, has the ability to influence FA bioconversion of the n-3 and n-6 FA pathway thereby increasing 22:6n-3 and possibly 20:5n-3 in rainbow trout and reducing ARA when fed VO-based diets. Furthermore, it seems as though petroselinic acid causes improved bioconversion to 22:6n-3 when the first Δ6 desaturase is bypassed. Further studies are needed to determine the mechanism of action that petroselinic acid has on FA bioconversion.

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DEDICATION

To my grandparents:
Bill and Netty Slugoski and Ozzy and Irene Randall
Thank-you for always watching over me.

I dedicate this thesis to my parents:
Jeanette and Neil Randall
Who taught me to strive for greatness and never give up!

Kyla Randall
Saskatoon, Saskatchewan
March 2011

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ABBREVIATIONS

ACO	Acyl coenzyme A oxidase
ADFI	Average daily feed intake
ADG	Average daily gain
ALA	α-linolenic acid
ARA	Arachidonic acid
ASP	Acid soluble products
BSA	Bovine serum albumin
CP	Crude protein
CPT1	Carnitine palmitoyl transferase I
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EF1A	Elongation factor 1a
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
ETIF3	Eukaryotic translation initiation factor 3
FA	Fatty acid
FAME	Fatty acid methyl ester
FBS	Foetal bovine serum
FCR	Feed conversion ratio
FFA	Free fatty acid

FM	Fishmeal
FO	Fish oil
HEPES	Phenylethylamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LO	Linseed oil
MG	Monoglyceride
MUFA	Monounsaturated fatty acid
OA	Oleic acid
PA	Petroselinic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PL	Phospholipid
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acid
SDA	Stearidonic acid
SEM	Standard error of the mean
SFA	Saturated fatty acid
SGR	Specific growth rate
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerol
TLC	Thin-layer chromatography
WBFA	Whole body fatty acid

VO	Vegetable oil
Δ5	Delta 5 desaturase
Δ6	Delta 6 desaturase

1 INTRODUCTION

Modern western diets are widely acknowledged to have an unhealthy balance of n-6 and n-3 fatty acids (FA; Simopoulos, 2011). Simopoulos (2011) reported that during the Palaeolithic period, n-6 to n-3 ratios were around 0.79:1, however current diets within Canada, the United Kingdom, northern Europe and the United States being very high in n-6 and deficient in n-3 FA, an n-6:n-3 ratio of 15-16:1. But, in India the ratio is even higher at 38-50:1. These values reflect the increased consumption of saturated n-6 fats from processed foods and vegetable oils (VO). This spike has resulted in increased incidence of cardiovascular disease, diabetes and obesity in humans (Kris-Etherton *et al*., 2000, 2002), all of which have been shown to decrease with increased consumption of n-3 rich foods. Fish are a primary source of n-3 FA in the human diet and the increased demand for n-3 FA has been associated with an increased demand for fish. However, this increased demand cannot be met by existing wild fish sources as they are gradually declining due to overfishing. This, in turn, has increased the importance of the global aquaculture industry to produce more fish for human consumption and increase the levels of n-3 FA in human diets.

The aquaculture industry is now the fastest growing animal production sector and is currently growing at an average annual rate of 6.9 % (Shepherd *et al*., 2005; FAO, 2011). This exponential growth in aquaculture has resulted in a concomitant growth of aquaculture feed production and caused further demand for the primary ingredients, fishmeal (FM) and fish oil (FO), used in diets for carnivorous fish species. The world's demand for FO already exceeds supplies and by 2020, the same will be true for FM. The imminent scarcity of these primary ingredients amplifies the necessity of finding a replacement for FM and FO. Until this dilemma

is solved, the sustainability of aquaculture is in jeopardy. With increased cost of these products along with the decline of wild fish stocks, consumers and producers are looking for low cost alternatives such as plant ingredients in order to maintain the sustainability of the industry.

However, in the case of FO, studies have shown that fish fed plant oil diets are low in n-3 FA (Bell *et al.,* 2003a, 2003b, 2004; Tocher *et al.,* 2002) thus reducing its nutritional value. In order to provide a health beneficial food source to consumers, alternative oil sources need to be established. This thesis will therefore focus on the problem of replacing FO in aquaculture feeds and ways in which to increase the levels of long-chain polyunsaturated (LC-PUFA) in aquaculture fish products.

2 LITERATURE REVIEW

2.1 DEVELOPMENT OF MODERN AQUACULTURE

Modern aquaculture encompasses all aspects of production, processing and marketing of aquatic plants and animals from fresh, brackish, and salt waters (Barnabé, 1990; Lucas, 2012). The practice of aquaculture is believed to have originated in China over 4000 years ago with carp species. It began with simple designs integrating ponds and dugouts with single species and eventually flourished into production with mixed species. This method of fish culture ultimately spread across Europe; however, it was not until the industrial revolution, during the nineteenth century, that aquaculture became a more intensive and productive production sector (Nash, 2011).

Scientific advances began to put forth improvements in rearing and management techniques, including artificial insemination for ease of international egg shipment, improved production facilities, nutrition and harvesting for both fresh and saltwater fish species. These

advancements helped form the aquaculture industry into what we know it as today. By the late 1950's, global aquaculture production was roughly 640,000 metric tonnes and rising rapidly (FAO, 2011). Feed formulations changed from a fairly basic design utilizing a few ingredients to a highly advanced mix using numerous ingredients with the inclusion of vitamins and minerals. Pasteurization and drying of feeds helped decrease the risk of microbial contamination and increased shelf life. This was the birth of pelleted feeds, a milestone for the aquaculture industry, which positively influenced feed conversion and reduced overall costs (Nash, 2011). The mid 1960's to mid 1970's brought the development of plastics, fibreglass and handheld water quality testing equipment, which allowed global aquaculture to expand into a safe, more productive industry with infinite options for fabricating tanks and hatchery systems. Due to the improved conditions, world aquaculture production jumped to over 2.5 million metric tonnes by mid 1970 (Table 2.1) and further increased to over 78 millions metric tonnes by 2010 (FAO, 2010; 2011). Production by region is dominated by Asia, which accounts for over 88 % of total production, whereas no other continent contributes more than 5 % of total world production in 2008 (Table 2.1). The FAO (2011) further reports that during the period of 1970-2008, food fish production increase at an average annual rate of 8.3 %, while world population grew at an average of 1.6 % per year, meaning that the average per capita annual food supply of food fish from aquaculture for human consumption increased ten times since 1970 at a rate of 6.6 % per year. In contrast, Canada was only contributing 2,700 metric tonnes to aquaculture production in the 1950's, which increased to 40,000 metric tonnes by the 1990's and over 160,000 by 2010.

Table 2.1 World production of aquaculture: quantity and percentage by region

Country		1970	1980	1990	2000	2006	2008
Africa	Tonnes	10 271	26 202	81 015	399 788	754 406	940 440
	%	0.40	0.60	0.60	1.20	1.60	1.80
America	Tonnes	173 491	198 850	548200	1 422, 637	2 367 320	2 405 166
	%	6.80	4.20	4.20	4.40	5.00	4.60
Asia	Tonnes	1 786 286	3 540 960	10 786 53	28 400 213	41 860 117	46 662 031
	%	69.60	75.20	82.50	87.60	88.40	88.80
Europe	Tonnes	510 713	770 200	1 616 287	2 702 160	2 209 097	2 366 354
	%	19.90	16.40	12.40	6.40	4.70	4.50
Oceania	Tonnes	8 421	12 224	42 005	121 312	160 126	172 214
	%	0.30	0.30	0.30	0.40	0.30	0.30
World	Tonnes	2 566 882	4 705 841	13 074 100	32 416 110	47 351 066	52 546 200

[1]Data modified from FAO (2010) and does not include aquatic plants

2.1.1 *Salmonid aquaculture production*

Salmonids are part of the Actinopterygii or ray-finned fish Class and the Family Salmonidae, which includes trout, salmon, char, freshwater whitefish and grayling (Nelson, 1984). They have an anadromous life cycle, meaning that they spend the majority of their life in marine waters and migrate to spawn in freshwater (Steelhead trout), though some salmonids can be non-anadromous, such as rainbow trout (*Oncorhynchus mykiss*), which spend their whole life cycle in freshwater. During the life cycle of a salmonid there are a series of changes from egg to adult fish, however, the timing of these changes vary among species (Hirschi, 2001). The life cycle begins in freshwater and the stages are as follows: egg, alevin (newly hatched), fry (yolk sac absorbed), parr/fingerling (juvenile fish with distinctive markings), smolt (parr/fingerlings that have undergone smoltification) and mature adult. In order for the salmonid to successfully migrate from salt to freshwater it must undergo a physiological, biochemical, morphological and behavioural change called smoltification (Folmar and Dickoff, 1980). During this process there are a series of changes within the fish regarding, but not restricted to, osmoregulation, lipid metabolism, oxygen transport, buoyancy, growth, colour (dark pigment to silver), shape,

rheotaxis (guided movement toward a stimulus) and schooling behaviour (Folmar and Dickoff, 1980; Stefansson *et al.,* 2008). The most significant of these changes is osmoregulation, where freshwater fish must counteract the passive gain of water and loss of ions through the production of dilute urine and actively taking up ions across the gills.

The Family Salmonidae makes up the majority of the cold-water species in the global aquaculture industry and accounted for 118,000 metric tonnes by the mid 1970's and further increased to over 2.5 millions metric tonnes by 2010 (FAO, 2011). Salmonid production occurs mainly in Chile, Norway, Scotland and Canada but production takes place in throughout Europe, Asia and the United States. Canadian salmonid production was only 30,000 metric tonnes in 1990, and has increased to only over 122,000 metric tonnes by 2010 (FAO, 2011). Overall, aquaculture production in Canada, which includes salmonids, has started to decline and has remained constant at just below 160,000 metric tonnes, whereas global levels are rising rapidly.

2.2 FISH OIL PRODUCTION

Fish oil is the primary source of LC-PUFA, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), and is known to have health-beneficial properties (Simopoulos, 1991, 1999; Kris-Etherton *et al*., 2000). It is formed during the process of converting raw whole fish, mainly pelagic fatty fish found in temperate and subtropical oceans such as Peruvian anchovy, mackerel, capelin, menhaden and herring, (De Silva *et al.,* 2011) into a stable, value-added product by a method called rendering. Fish oil is generally produced by a process called wet reduction, whereby raw fish material is hashed (cut into pieces), cooked, pressed, separated, polished, and stored. However, prior to storage, an optional carbon treatment can be performed on the separated product to reduce levels of dioxins, furans and/or

polyaromatic hydrocarbons (Bimbo, 2000). Fish oil can be further refined to remove containments through neutralization, bleaching, and deodorization. Refined FO is most commonly used in aquaculture and fish farming since, unrefined FO contains higher levels of heavy metals, polychlorinated biphenyls, dioxins and furans, which may accumulate in aquaculture-reared fish (Makrides *et al.,* 2005) and cause health issues. Oil can be extracted from whole fish, liver, and offal and holds importance not only in aquaculture (Figure 2.1) but also in livestock production of ruminants (Dairy; Santos *et al.*, 1998; Ponnampalam *et al.,* 2002) and non-ruminants (Swine; Wood *et al.,* 1999; Hallenstvedt *et al.,* 2010; Poultry; López-Ferrer *et al.,* 1999; Shin *et al.,* 2011), the pet food industry (De Silva and Turchini, 2008), the pharmaceutical industry (Donadio and Grande, 2004), and most importantly for human consumption (Simopoulos, 1991, 1999; Kris-Etherton *et al*., 2000). The wide spread use of FO is the centre of many debates regarding the quantity of FO used for sources other than for human consumption (De Silva and Turchini, 2008; Wijkström, 2009). De Silva *et al.* (2011) stated that the use of FO in aquaculture is the key impediment on future growth and sustainability for the industry. This is due to cost, fluctuations in wild fish stocks, and the growing market demand due to the importance of health-beneficial LC-PUFA that fish these species possess. With the decline in wild fish stocks, animal production industries are searching for cost effective, readily available alternative oil sources.

Annual FM and FO production worldwide has remained relatively stable since the early 1960's, reflecting the overall stability of global pelagic fish (Figure 2.2, IFFO, 2008). Total production of FO is approximately 1.4 million tonnes per year (Shepherd *et al*., 2005) and this level of production is expected to remain constant or slightly decrease in the future. The International Fishmeal and Fish oil organization (IFFO) (2008) stated that the allocation of FO in

the aquaculture sector jumped from 16 to 98 % from 1990 to 2010. Therefore, almost all produced FO is currently used by aquaculture, making it critically important that alternative sources of oils be developed. De Silva *et al.,* (2011) reported that 100 kg of raw fresh fish material yields an average of 20 kg of FM and 5 kg of FO after processing and roughly 25 % of the worlds capture fisheries are utilised by the reduction industry to make FO and FM.

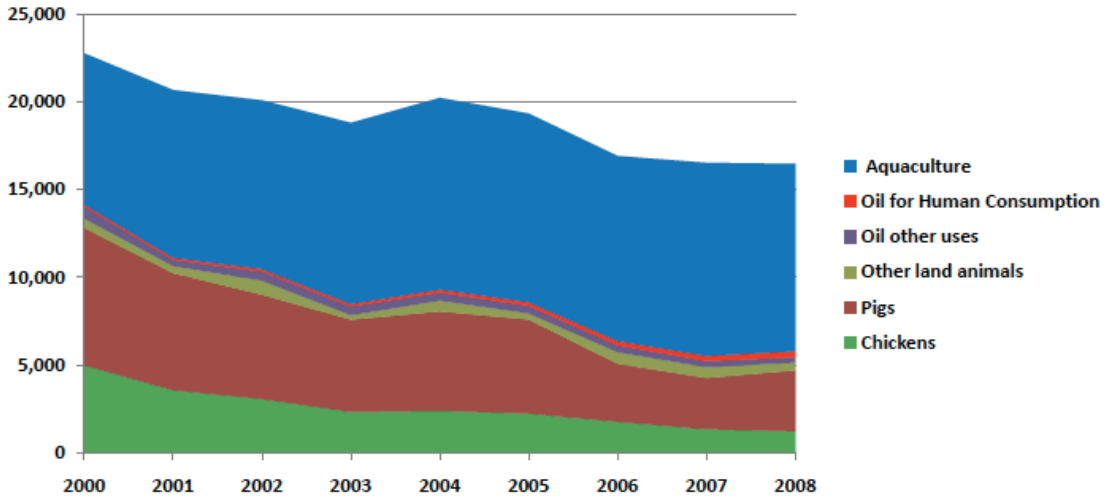

Figure 2.1 Allocation of whole rendered fish (tonnes ,000), IFFO (2008)

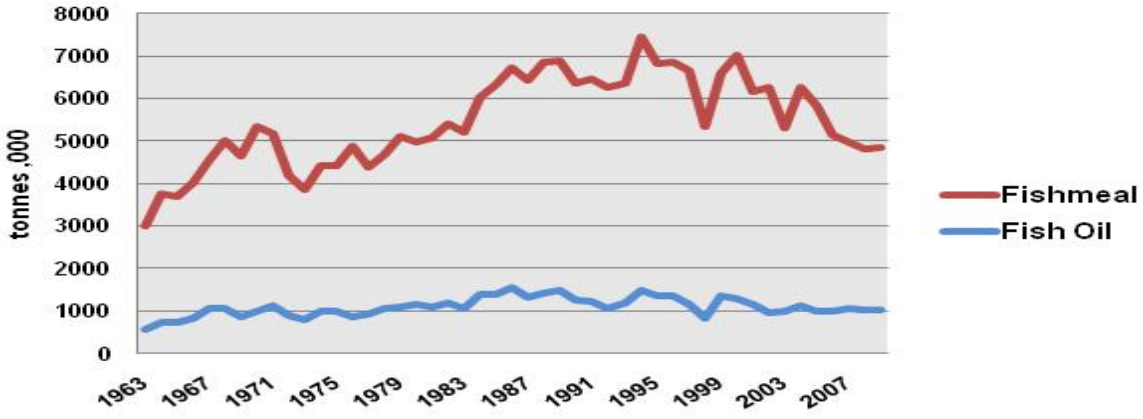

Figure 2.2 Global trends of fishmeal and fish oil production, IFFO (2008)

2.3 DIETARY LIPID AND ESSENTIAL FATTY ACID (EFA)

Dietary lipids are hydrophobic compounds that dissolve in non-polar solvents, such as hexane, chloroform, and methanol but not in water (Gurr and Haywood, 1991). They include, but are not limited to, fat-soluble naturally occurring compounds such as oils, waxes, sterols, triacylglycerols (TAG) and phospholipids (PL). They are an inexpensive energy source that contains roughly 2.25 times more energy per unit weight than carbohydrate or protein (Honeyboune *et al*., 2004; Cole, 1966). Their main functions include energy production through TAG, structural components of cell membranes, cellular metabolism including absorption of fat-soluble vitamins, signal transduction and inflammatory response through hormones such as steroids and prostaglandins and are sources of essential fatty acid (EFA, NRC, 1993; Halver 1980; Lovell, 1998). All of these factors work together to provide proper growth, maintenance, metabolic functions and homeostasis of fish. Additionally lipid added to a diet imparts flavour and texture for better efficiency, processing and palatability.

Dietary lipid consists of an aliphatic hydrocarbon chain of varying length, which terminates with a carboxylic acid, causing the molecule to have polar, hydrophilic end and a non-polar, hydrophobic end, which is insoluble in water. Fatty acids can either be saturated, containing no double bonds, such as palmitic acid found in palm oil, monounsaturated, containing one double bond, such as oleic acid (OA; 18:1n-9) found in canola oil, or unsaturated, containing 2 or more double bonds, such as the EFA linoleic and linolenic acid found in plants and 20:5n-3 and 22:6n-3 found in fish, algae and bivalves (Tocher, 2003). The notation of FA is determined by numbering either from the methyl or carboxyl terminal end of the carbon chain. In fish nutrition it is more common to number from the methyl terminal end or more commonly

called the omega end. Fatty acid notation includes a few basic steps:

1. The first number indicates the number of carbon atoms
2. The second number, following the colon, is the number of double bonds
3. The third number, designated as (n-), is the number of carbon atoms between methyl terminal and the first double bond

For example a FA with the notation 18:3n-3 would contain 18 carbon atoms, 3 double bonds and the first double bond would be located at the 3rd carbon from the omega carbon. This dictates that it is an n-3 FA.

All vertebrates, including freshwater fish, lack Δ12 and Δ15 n-3 fatty acyl desaturases and therefore, cannot synthesise the base LC-PUFA linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) *de novo* from the monoene, 18:1n-9 (Norsker and Støttrup, 1994; Tocher *et al*., 2004; Rinchard *et al*., 2007; Tocher, 2009). Because of this, these FA must be obtained in the diet to maintain normal growth and development and are therefore termed as "essential fatty acids". This term however has been up for debate due to its lack of clarity. A review by Cunnane (2003) illustrates that 18:2n-6 and 18:3n-3 can be synthesised in rats *in vivo* by bioconversion of shorter FA such as tetradecadienoate (14:2n-6), tetradecatrienoate (14:3n-3), hexadecadienoate (16:2n-6), and hexadecatrienoate (16:3n-3), respectively. Nevertheless, salmonids are unable to synthesie 18:2n-6 and 18:3n-3 *de* novo and therefore these FA must be provided in the diet, however, LC-PUFA can be synthesised in salominds from 18:2n-6 and 18:3n-3 through a series of desaturation and elongation steps, which will be discussed further in section 2.6. However, marine fish are unable to synthesise LC-PUFA from 18:2n-6 and 18:3n-3 due to low Δ6 desaturase activity, which is considered to be the rate limiting factor in the synthesis of LC-PUFA (Tocher *et al.,* 2006).

When there is absence of EFA in the diet it can lead to deficiency symptoms that, in humans, include dry skin, hair loss, depression and other forms of mental illness as well as heart

disease (Wene *et al.,* 1975). In fish, deficiencies can trigger reduced growth, survival and reproductive potential, fatty liver, intestinal steatosis, symptoms such as fin rot, loss of consciousness (shock syndrome) and spinal cord abnormalities (lordosis; Wantanabe *et al*., 1988; Ruyter and Thomassen, 1999; Tocher, 2003, 2009). Furthermore, deficiencies in diet FA composition can affect fish tissue FA composition, causing undesirable FA profiles for human nutrition (Fonseca-Madrigal *et al*., 2005).

2.4 LONG CHAIN N-3 FATTY ACIDS AND THEIR HEALTH BENEFITS

Dietary lipids can be broken down into several groups including, but not limited to, n-3, n-6 and n-9 FA depending on the location of the double bond within the carbon chain. The most commonly talked about FA are 18:3n-3, 18:2n-6, arachidonic acid (ARA; 20:4n-6), 20:5n-3 and 22:6n-3. n-3 and n-6 FA such as 18:3n-3, 18:2n-6 and 20:4n-6 can be are found in nuts, animal and plant oils such as flax, canola, soybean, safflower and corn (Rinchard *et al*., 2006; Harper and Jacobson, 2001) and 20:5n-3 and 22:6n-3, can be found in algae, seaweed, oily fish and invertebrates (Kris-Etherton *et al.,* 2002). n-9 FA can be found in fruits, nuts, seeds, animal and plant oils, the highest being in olive, canola, peanut and grapeseed (Batista *et al.,* 1999; Sanaiotti *et al.,* 2008).

Omega-3 FA are found in almost all living organisms, but primarily in marine and plant oils. Omega-3 FA from fish are unique, as they contain important LC-PUFA, 20:5n-3 and 22:6n-3. Extensive research has reported that these LC-PUFA help lower the risk of cardiovascular disease, stroke, autoimmune and inflammatory diseases, cancer, dementia and Alzheimer's disease and benefit the immune and nervous systems (Harold and Kinsella, 1986; Nettleton, 1995; Morris *et al*., 1995; Kris-Etherton *et al.,* 2002, 2003; Hooper *et al.,* 2006; Fernández *et al.,*

1999a; Singer *et al.,* 2008; Nestel, 2000). Omega-3 rich VO, on the other hand, do not naturally contain any level of 20:5n-3 and 22:6n-3 but a variety of C18-22 FA, especially 18:2n-6 and 18:3n-3 (Table 2.4).

There is a general misconception among consumers that n-3 FA are primarily needed for elderly people or for those with existing heart disease, however this is not the case, as the importance of high n-3 FA in the diet begins in foetal development (Nettelton, 1991; Simopoulos, 1991). Extensive research has proven that, during the 3rd trimester of pregnancy and further until 18 months postpartum, LC-PUFA, especially 22:6n-3, become incorporated into the membrane PL of the retina and brain (Clandinin *et al*., 1980; Craig-Schmidt *et al*., 1996; Hoffmann *et al*., 1993, Walker, 1967; Hrboticky *et al*., 1989; Martinez and Ballabriga, 1987). Health and Welfare Canada (1990) suggests that pregnant women in the age range of 19 to 49 years of age should consume a minimum of 1.36 g d^{-1} and 1.26 g d^{-1} of 20:5n-3 and 22:6n-3 respectively, during the 2nd and 3rd trimesters to ensure proper development of the foetus. The American Dietetic Association recommendations are slightly lower stating consumption of 1 g d^{-1} of 20:5n-3 and 22:6n-3: the equivalent to one 3oz portion of farmed or wild Atlantic salmon (*Salmo salar*) or farmed rainbow trout (Table 2.4) (Kris-Etherton *et al*., 2002). The most prominent issue related to consuming these heath-beneficial LC-PUFA is the ratio of n-6 to n-3 FA. Currently the ratio of consumed n-6:n-3 FA is substantially higher then that reccommened by American Dietetic Association.

Table 2.2 Amount of EPA + DHA in fish (g 3oz $^{-1}$)

Food	***EPA + DHA Content, g/3-oz Serving Fish or g/g Oil***
Salmon, Atlantic farmed	1.09-1.83
Salmon, Atlantic wild	0.9-1.56
Trout, Rainbow farmed	0.98
Trout, Rainbow wild	0.84
Herring, Pacific	1.81
Herring, Atlantic	1.71
Tuna, Fresh	0.24-1.28
Tuna, white canned	0.73
Halibut	0.4-1.0
Cod, Pacific	0.13
Cod, Atlantic	0.24

Modified from Kris-Etherton *et al*., 2002

2.5 BALANCE OF N-6:N-3 FATTY ACID RATIO

Maintaining the balance between n-6 and n-3 FA is essential for maintaining the balance between inflammation and immunity within the body, regardless of species. The optimal n-6 to n-3 ratio is 1-2:1 (Crawford, 1968; Eaton *et al.,* 1998; Simopoulos, 1991, 1999; Kris-Etherton *et al*., 2000). Several studies suggest that during the late Palaeolithic period dietary n-6:n-3 ratios were 0.70 g d^{-1} (LA:ALA), whereas in the current Western world dietary ratios are reported to be at levels of 18.75 g d^{-1} (Simopoulos, 1998). In 1985, people living in the United States had consumed n-6 and n-3 FA in an average ratio of 12.4:1, though this dropped to 10.6:1 by 1994 (Cordain *et al.,* 2005; Kris-Etherton *et al.,* 2000), yet increased again to 16:1 within a few years (Simopoulos, 2008) due to advancements in food technology and economic stimulus. This high consumption of n-6 fats has resulted in increased levels of cardiovascular disease, inflammatory and autoimmune diseases and cancer (Simopoulos, 2002) due to increased production of biologically active eicosanoids produced from 20:4n-6, shifting the physiological state from pro-thrombotic to pro-aggregatory (Simopoulos, 2006). Increasing levels of n-3 FA suppresses these effects and reduces the incidence of these conditions.

2.6 FATTY ACID METABOLISM

2.6.1 *Fatty acid digestion, absorption and storage*

During the process of lipid digestion FA are liberated by hydrolysis of the ester bond in TAG. Since lipids are insoluble in water, TAG must be physically hydrolysed into smaller particles to expose the surface area for attack by digestive enzymes in order for further utilisation to occur. This is achieved through the emulsification of the TAG with mucous, hydrochloric acid and bile salts in the small intestine (Barnes, 2006; Gropper *et al.*, 2009) allowing pancreatic lipase to gain access to the fat molecules, forming mixed micelles. As the chyme passes into the duodenum, cholecystokinin stimulates the gallbladder and pancreases to release bile and pancreatic lipase, respectively, which aids to emulsify the fat (Barnes, 2006). After the lipid has been emulsified within the small intestine, intestinal lipases further degrade TAG by splitting the FA at positions 1 and 3, leaving behind 2-monoglyceride and two free FA (FFA; Figure 2.3), both of which can efficiently diffuse into the enterocyte, which can later be utilised by the body (Shi and Burn, 2004).

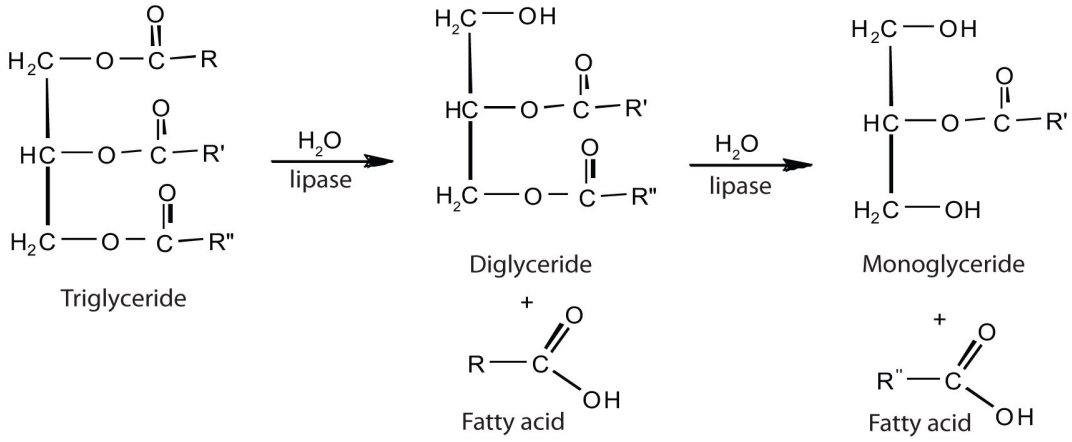

Figure 2.3 Breakdown of triglycerides by intestinal lipase, Ball *et al.* (2011)

Absorption of lipid occurs in the small intestine. Short-chain and medium-chain FA, ≤ 12 carbons, are directly absorbed through the villi of the intestinal mucosa bound to albumin and enter into the bloodstream where they are incorporated into TAG. Medium-chain FA, > 12 carbons, on the other hand, are absorbed mainly in pyloric caeca leaving long-chain FA to be absorbed primarily in the mid intestine (Røsjø *et al*., 2000). Within each villus there are a series of lymph vessels called lacteals and capillaries. The lacteals absorb the FA and glycerol into the lymphatic system, which drains into the bloodstream (Røsjø *et al*., 2000). However, epithelial cells must re-synthesise the monoglycerides and long-chain FA into TAG within the midgut in order for incorporation into the lymphatic system. This is performed *via* small spheres called chylomicrons, large molecules of lipoprotein, cholesterol and PL, used for transport of dietary lipid to liver and adipose tissue (Gropper, Smith and Groff, 2009). These chylomicrons are then transported across the basolateral membrane into the lymphatic system (Stevens and Hume, 1995). Fat-soluble vitamins, long-chain alcohols, and other lipids also appear to be incorporated into chylomicrons to enter the lymphatic system. Once in the bloodstream FA are transported to the membrane of adipose or liver cells where they can be stored or oxidised for energy. Once the lipid is delivered to the liver they are in the form of chylomicron remnants, which are formed when chylomicrons breakdown following lipolytic action (Røsjø *et al*., 2000). The liver has the ability to catabolise exogenous lipids that are delivered to it in the form of these remnants (Gropper, Smith and Groff, 2009). The chylomicron remnants attach to binding sites with lipoprotein lipase and deliver FFA, diglycerides, monoglycerides, PL and cholesterol to tissues throughout the body (Gropper, Smith and Groff, 2009). In mammals, lipids are mainly deposited in adipose tissue, however in fish, lipid storage sites vary between species but include liver, muscle, and mesenteric tissue (Henderson and Tocher, 1987; Sheridan, 1988; Zhou *et al.,* 1995,

1996). In salmonids, the primary storage sites for lipids are visceral adipose tissue and adipocytes located within the connective tissue of muscle, called myosepta (Polvo and Ackerman, 1992; Rosen and MacDougald, 2006). On the other hand, white fish, such as cod, store fat primarily in the liver (30-50 %) and less than 1 % in muscle (Zhou *et al.,* 1995, 1996).

2.6.2 *Desaturation, elongation and β-oxidation*

Lipid metabolism is similar among freshwater and marine fish, however marine fish have limited Δ6 desaturase enzyme activity (Tocher *et al.,* 2006). As previously mentioned, n-3 FA, including 20:5n-3 and 22:6n-3, are essential in fish as their cells cannot synthesise 18:3n-3 and 18:2n-6 *de novo* and they must, therefore, be provided in the diet (Ruyter *et al*., 1999, 2000; Sprecher, 2000; Hastings *et al*., 2001; Simopoulos, 2002; Tocher, 2003; Turchini *et al*., 2006). The n-3 and n-6 FA pathways share the same enzymes, creating competition between the FA for the desaturation enzymes. However, it has been reported that n-3 FA are the preferential substrate for both Δ4 and Δ6 desaturases compared to n-6 FA (Hauge and Christoffersen, 1984; Simopoulos, 2008). Overall, the relative activities of these enzymes are what determine the relative amounts of products formed.

The FA biosynthetic pathways in fish consist of a series of desaturation and elongation steps (Figure 2.4; rainbow trout, Buzzi *et al.,* 1996, 1997; mammals, Guillou *et al.,* 2010). First 18:3n-3 is converted to stearidonic acid (SDA; 18:4n-3) by Δ6 desaturase, which is the rate-limiting step in the production of n-3 PUFA (Turchini *et al.,* 2006; Turchini and Francis, 2009, Glencross, 2009, Guillou *et al*., 2010). There is also an alternative pathway that bypasses Δ6 desaturase, where 18:3n-3 is elongated *via* Elovl 5, by addition of dicarbon units, to 20:3n-3 followed by the addition of a double bond *via* Δ8 desaturase forming 20:4n-3 (Turchini *et al.,*

2006; Turchini and Francis, 2009, Guillou *et al*., 2010). Subsequently, 18:4n-3 is elongated *via* Elovl 5 to 20:4n-3 followed desaturation by Δ5 desaturase producing 20:5n-3. 20:5n-3 is then elongated by Elovl 2 and 5 to 22:5n-3, which is further elongated to 24:5n-3. The final Δ6 desaturase event converts 24:5n-3 to 24:6n-3, which is then chain shortened to 22:6n-3 by peroxisomal retro-conversion (Turchini *et al.,* 2006; Turchini and Francis, 2009, Glencross, 2009).

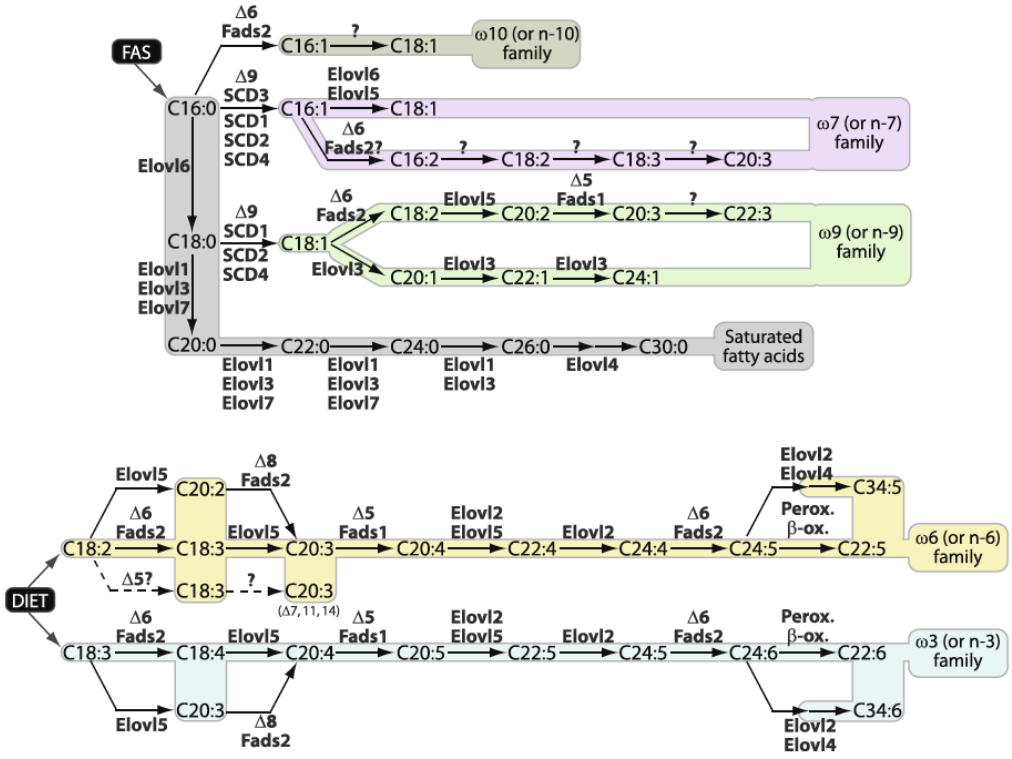

Figure 2.4 Long chain and very long chain fatty acid biosynthesis in mammals, Guillou *et al*. (2010)

For the n-6 biosynthetic pathway, the first step is the desaturation of 18:2n-6 by Δ6 desaturase producing 18:3n-6. An alternative pathway also exists for the n-6 pathway, where by Δ6 desaturase is bypassed and 18:2n-6 is elongated to 20:2n-6 *via* Elolv 5, which is then

desaturated to 20:3n-6 *via* Δ8 desaturase (Turchini and Francis, 2009, Glencross, 2009, Guillou *et al.,* 2010). The enzyme Δ5 desaturase then adds an additional double bond to 20:3n-6 forming 20:4n-6. 18:3n-3 and 18:2n-6 share the same desaturation enzymes within their FA pathway, making competition of n-3 and n-6 FA for these enzymes inevitable (Brenner, 1977; Sprecher, 2002; Portolesi *et al*., 2007). The FA that becomes desaturated depends on affinities of the FA for the Δ6 desaturase as well as their proportions (Brenner, 1977). If competition favours one pathway over the other, eicosanoid production and subsequent inflammatory responses can be altered.

2.6.3 *Fatty acid regulation through transcription factors*

The activities of Δ5 and Δ6 desaturase, in the n-3 and n-6 FA pathways are nutritionally regulated (Zheng *et al*., 2005ab; Cho *et al*., 1999a; Cho *et al*., 1999b; Seiliez *et al*., 2001). A study by Zheng *et al*. (2005a, 2005b) showed that the expression of both Δ5 and Δ6 desaturase genes and the activity of the LC-PUFA biosynthetic pathway were higher in fish fed VO compared to fish fed FO. Mozaffarian *et al.,* (2005) reported that high tissue levels of 20:5n-3 and 22:6n-3 inhibited the expression of Δ5 and Δ6 desaturases and this negative feedback mechanism decreases the production of LC-PUFA from 18:2n-6 and 18:3n-3 in the diet. This suggests that in human diets, plant-based 18:3n-3 may only influence heart related disease when intake of seafood-based, 20:5n-3 and 22:6n-3 is low (<100 mg d^{-1}) (Mozaffarian *et al*., 2005; Siscovick *et al*., 2003).

Nutritional regulation of Δ5 and Δ6 desaturase appears to be accomplished through changes in the activities and abundances of several nuclear receptor proteins that function as transcription factors, specifically peroxisome proliferator-activated receptors (PPAR) and sterol

regulatory element-binding proteins (SREBP). PPAR belong to the steroid/thyroid/retinoid nuclear receptor superfamily and are lipid activated transcription factors that regulate the expression of genes (Lemberger *et al*., 1996; Berger and Moller, 2002). PPAR consist of three distinct subtypes, which are expressed in various tissues, α, ß (also expressed as δ) and γ. PPARα is present in heart and skeletal muscle but mainly in liver where it has an important role in the regulation of nutrient metabolism, including FA oxidation, gluconeogenesis, ketone body synthesis and amino acid metabolism (Ferré, 2004; Kersten, 2002; Sharma, and Staels, 2007). PPARβ is expressed all throughout the body and activates gene expression resulting in FA oxidation where PPARα is absent. Lastly PPARγ is expressed in the lower intestine, immune cells and mainly in adipose tissue where it stimulates adipogenesis, lipogenesis and glucose utilisation (Ferré, 2004; Kersten, 2002). The PPAR family is grouped together on the basis of structural similarities consisting of a central DNA-binding domain containing two zinc fingers and a large C-terminal domain that binds ligands (Figure 2.5; Kersten, 2002; Ferré, 2004). PPAR regulate target gene expression by binding to specific peroxisome proliferator response elements (PPRE) in enhancer sites of regulated genes (Figure 2.6). Several studies have shown that PPAR bind to PPRE on the DNA AGGTCA-AGGTCA sequence, which is a direct repeat scattered throughout the genome containing a single nucleotide spacer, as obligate heterodimers with 9-*cis*-retinoic acid receptor or retinoic x receptor alpha (Figure 2.5; Ferré, 2004; Berger and Moller, 2002; Kersten, 2002). The confirmation of a PPAR is altered and stabilised such that a binding cleft is created and recruitment of transcriptional co-activators occurs. The result is an increase in gene transcription (Berger and Moller, 2002). These transcription factors are activated by different factors. For example, PPARγ is activated by prostaglandins, leukotrienes and anti-diabetic thiazolidinediones and affects the expression of genes involved in the storage of

the FA. PPARß, on the other hand, is only weakly activated by FA, prostaglandins and leukotrienes and has no known physiologically relevant ligand. Dreyer *et al.* (1993) and Huffman *et al.* (2004) described that LC-PUFA, 20:5n-3 and 22:6n-3 were more potent ligands than saturated FA.

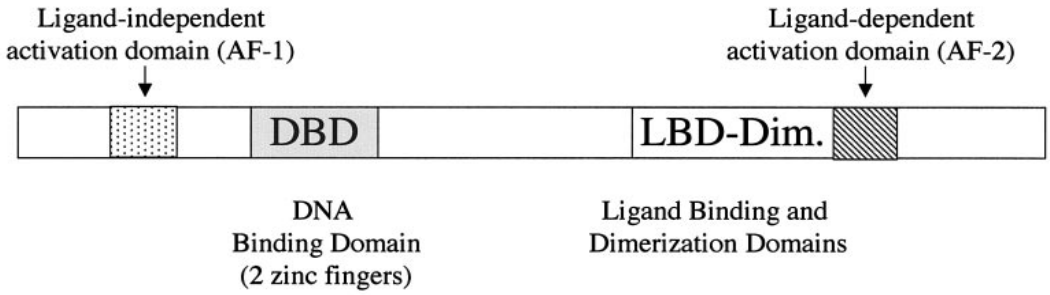

Figure 2.5 Structure of PPAR, Ferré (2004)

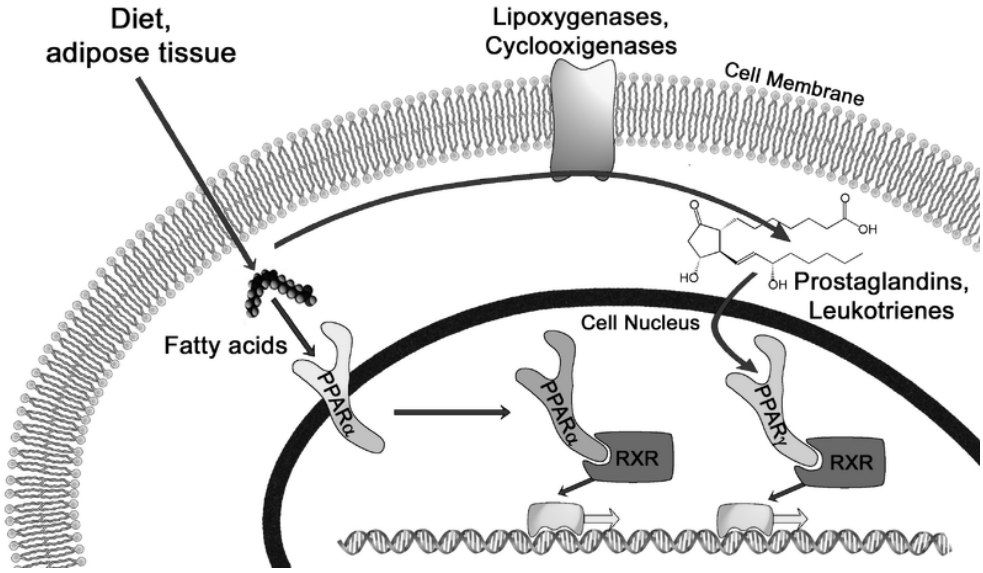

Figure 2.6 PPAR pathway, Wikipedia contributors (2006)

SREBP, which are regulated by cholesterol, are another form of transcription factor of the basic helix loop-helix leucine zipper family, which bind to the sterol regulatory element (SRE) DNA sequence TCACNCCAC (Nakamura and Nara, 2004). There are two isoforms of

SREBP, SREBP-1 and SREBP-2. SREBP-1 has two subforms SREBP-1a and SREBP-1c. SREBP-2 activates the transcription of genes involved with cholesterol synthesis and metabolism, SREBP-1c targets genes for FA synthesis and is important as it mediates the PUFA inhibition of Δ6 desaturase in liver (Nakamura and Nara, 2004) and SREBP-1a induces both functions. SREBP is positioned in a hairpin needle fashion, meaning that the N-terminal and C-terminal domains loop into the cytosol. They are then separated by two transmembrane-spanning helices connected by one loop in the lumen of the endoplasmic reticulum or nuclear envelope (Kotzka *et al.,* 2004).

Proteolytic cleavage frees SREBP from its membrane bound state for movement through the cytoplasm to the nucleus. Once in the nucleus, SREBP can regulate gene transcription by binding to *cis*-acting elements denominated SRE that are found in the control regions of the genes that encode enzymes needed to make lipids (Figure 2.7, Brown and Goldstein, 1997). SREBP are localised in the endoplasmic reticulum, but in the event that the cell needs to synthesise cholesterol, the NH2-terminal portion of these proteins is cleaved by two specific proteases, and then travels into the nucleus to function as a transcriptional factor (Manzano *et al*., 2002). Sterols in turn inhibit the cleavage of SREBP and therefore synthesis of additional sterols is reduced through a negative feed back loop. The SREBP pathway is regulated by a cholesterol-sensing protein called SREBP cleavage activating protein (SCAP), which forms a complex with SREBP owing to interaction between their respective carboxy-terminal domains (Brown and Goldstein, 1997).

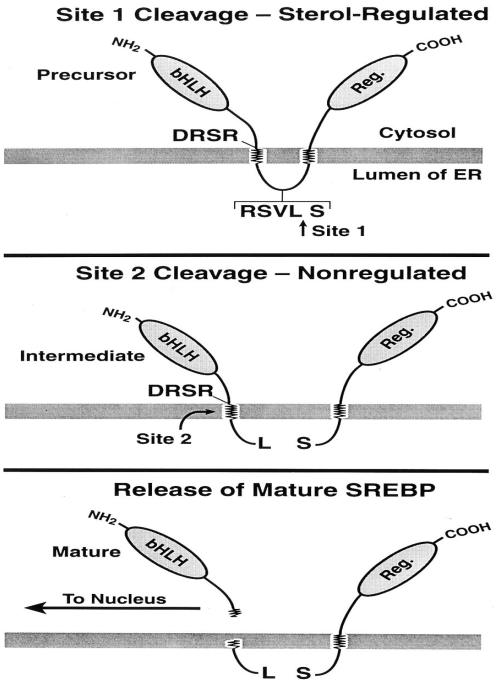

Figure 2.7 Model for Two-Site Proteolytic Cleavage of Membrane-Bound SREBP. bHLH, basic-helix-loop-helix-leucine zipper domain of SREBP; Reg., regulatory domain of SREBP, Brown & Goldstein (1997)

2.7 LIPID QUALITY

In order for the proper metabolism and utilisation of dietary FA to occur, lipid oxidation must be minimised. This can be achieved by removing the presence of activators such as metals, enzymes, debris/solids, heat, light and oxygen and storing the lipid or oil in well sealed, dark glass container with layers of inert gas. Antioxidants can also be added, where they act as a scavenger for free radicals and reduce the activity of reactive oxygen species. Overall, products of oxidation are monohydroperoxides, which are precursors of noxious odours and flavours that diminish the quality of the oils. Other volatile products of oxidation, which contribute to unwanted flavours, include ketones, alcohols, furan derivatives, alkynes, vinyl alcohols, as well as short-chain FA (Kanavouras *et al*., 2006).

Lipid oxidation is a degenerative process whereby free radicals take electrons from lipid, mainly unsaturated FA, as they contain multiple double bonds (Sherwin, 1978). Free radical chain reactions occur in three steps including: initiation, propagation and termination. In initiation, a FA radical is formed when a proton departs from the α-methylenic carbon in the unsaturated FA group of the fat molecule resulting in water and a FA radical (Shahidi and Wanasundara, 1992; Khal and Hildebrandt, 1986, Sherwin, 1978). Atmospheric oxygen, light and heat act as catalysts in this process (Fernández *et al*., 1986; Khal and Hildebrandt, 1986). During propagation, the FA free radical is oxidised, forming a lipid peroxy radical, which acts on another PUFA to generate hydroperoxides and another lipid alkyl radical (Piergiovanni and Limbo, 2009). Hydroperoxides, in the presence of high temperature or metals, are decomposed to alkoxy radicals which form short chain aromatic organic compounds such as alcohols, acids, ketones and aldehydes, which cause typical unpleasant and noxious odours and flavours (Piergiovanni and Limbo, 2009). This step destroys the nutritional value and palatability of the fats and oils. These free radicals are strong initiators and catalysts to further oxidation, which begins a self-perpetuating chain reaction until the lipid is completely oxidised or the reaction is terminated (Khal and Hildebrandt, 1986; Sherwin, 1978). Lastly, in the termination stage, two radicals react resulting in a non-radical species as seen in Figure 2.8. Antioxidants in the form of vitamin E, or α-tocopherol may also interfere, causing termination (Shahidi and Wanasundara, 1992; Piergiovanni and Limbo, 2009).

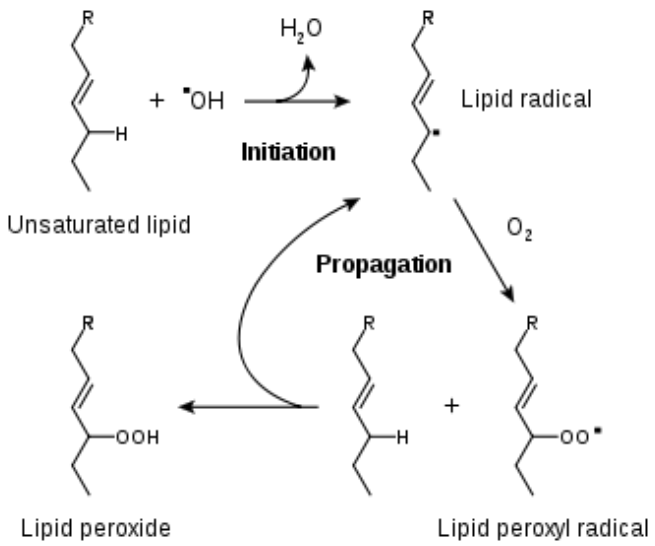

Figure 2.8 Lipid peroxidation and peroxide termination by antioxidant activity, Kelly *et al*. (1998) and Young and McEneny (2001)

2.8 STRATEGIES TO REPLACE FISH OIL WITH ALTERNATIVE OILS

2.8.1 *Single-cell marine oils*

Thraustochytrids, diatoms, microalgae and some marine bacteria are single-celled organisms that play a vital role in the nutrition of aquatic species as they make up the base of the entire aquatic food chain. Compared to terrestrial crops, single-celled organisms have a higher yield per hectare (Demirbas and Demirbas, 2010, Miller *et al.,* 2011) and are rich in 20:5n-3 and 22:6n-3 (FAO, 1996) making them desirable FO alternatives, yet the chemical composition varies depending on species and cultivation conditions, which can limit large-scale production. Microalgae biomass and live algae are primarily used as colouring agents and as a food source during the larval stages of some marine gastropods and fish, penaeid shrimp, zooplankton and during all life stages of marine bivalve molluscs (FAO, 1996), however they have also been utilised as a FO replacer in freshwater salmonid diets (Hertrampf and Piedad-Pascual, 2000).

Due to the high cost of production, risk of contamination, and varied food value, the use of microalgae has become limited in aquaculture production (FAO, 1996), and currently oil extracted from algae is not used in aquaculture (Miller *et al.,* 2011).

Currently the most promising single-celled FO alterative is thraustochytrids as they generate an abundance of n-3 LC-PUFA, especially DHA, and can be produced in large quantities (Millet *et al.,* 2011). Miller *et al.* (2007b) show that replacing FO with 100 %, 22:6n-3 rich, thraustochytrid (*Schizochytrium sp.* L) in Atlantic salmon diets caused a significant increase in 22:6n-3 (33.1 % of total FA) in white muscle tissue compared to FO fed fish. When thraustochytrid oil was mixed with palm oil at a 4:1 ratio it caused a significant increase in 22:6n-3 when compared to palm oil fed fish (11.3 to 9.8 %), however when compared to FO fed fish there was also a significant decrease but more comparable values (11.3 to 12.8 %). In an earlier study, Carter *et al.* (2003) reported no detrimental effects on performance from feeding 10 % thraustochytrids, however feeding FO still remained superior in providing high levels of 20:5n-3 and 22:6n-3.

2.8.2 *Terrestrial plant oils*

Terrestrial plant oils are the most inexpensive and sustainable method of replacing FO in aquaculture diets. However, terrestrial plant oils are devoid of LC-PUFA and contain varying amounts of n-3 and n-6 fatty acids (Table 2.5). Many of these oils have been investigated as replacements for FO (Table 2.6) in salmonids including, palm oil (Torstensen *et al.,* 2000; Bell *et al.,* 2002; Ng *et al.,* 2004) and soybean oils (Grisdale-Helland *et al.*, 2002; Rørå *et al.,* 2005), high n-3 FA flax/linseed oils (LO) (Tocher *et al.,* 2000; Bell *et al.,* 2003b, 2004a), canola/rapeseed (Bell *et al.,* 2001, 2003a, 2003b; Ng *et al.,* 2004; Torstensen *et al.,* 2004) and

camelina (Bell *et al.,* 2010; Atlantic cod, Morias *et al.,* 2011).

Crude palm oil contains no 18:3n-3 yet has exceptional oxidative stability due to its high concentration of saturated and MUFA, 16:0 (45%) and 18:1 (40%) respectively. As previously mentioned fish fillets FA composition mimics that of the oil fed, therefore when crude palm oil fully replaces FO in salmonid diets it causes increased deposits of saturated FA in the fillet thereby decreasing the health beneficial LC-PUFA, 20:5n-3 and 22:6n-3. Salmonids, being a cold-water species, require a PUFA rich diet in order to maintain membrane fluidity and provide adequate energy for daily activities. Despite this, Torstensen *et al.* (2000) and Bell *et al.* (2002) reported no negative effects on growth performance and feed efficiency when crude palm oil, replaced 25-100 % of FO in salmonid diets. However Bell *et al.* (2002) did report decreased lipid and FA digestibilities during low water temperatures in winter. The disadvantage to using crude palm oil is its affect on increasing fillet saturated FA. This can be altered by providing a washout period where the fish could be placed on a FO based diet during the last months of finishing prior to harvest.

Soybean oil is rich in 18:2n-6 (54 %), 18:1n-9 (24 %) and tocopherols but also low in 18:3n-3 (8 %). When salmonids were fed 50-100 % soybean oil no detrimental effects are seen on fish health, growth or feed efficiency (Grisdale-Helland *et al.*, 2002; Welker and Congleton, 2003; Sener and Yildiz, 2003; Ruyter *et al.,* 2006). Further, Rørå *et al.* (2005) saw no significant effect on liquid-holding capacity or texture of fresh and smoked Atlantic salmon fillets when 50 or 100 % soybean oil replaced FO, however smoking yield increased with inclusion of soybean oil. In all studies FA composition of the fillet reflected that of the oil fed and 20:5n-3 and 22:6n-3 were significantly lower in the 100 % soybean oil fed fish compared to the FO fed fish.

Canola is a major oilseed crop in the *Brassicaceae* family, which is known for its low erucic acid content. It is suitable for use as edible oil by humans and livestock and is also used for biodiesel. Canola oil is low in saturated FA containing less than 6 %, moderate in PUFA ranging from 25-40 % and high in MUFA at upwards of 65 %, when compared to other VO. Canola oil contains two important biologically active compounds: tocopherols, which are found at levels around 770 mg kg^{-1} oil and include α- (270 mg kg^{-1}) and γ- (420 mg kg^{-1}), and sterols, ranging from 0.7 to 1.0% and include β-sitosterol (52 %), campesterol (28 %) and brassicasterol (14 %; Gunstone and Harwood, 2007). These compounds increase oxidative stability thereby prolonging shelf life and have been reported to have positive effects on human plasma cholesterol levels (Reviewed by Moghadasian and Frochlich, 1999). There have been many advances, despite its controversy, to genetically modify canola to contain increased levels of 18:1n-9 and 18:4n-3 (Scarth and McVetty, 1999; Ursin, 2003; Gillingham *et al.,* 2011). These advancements would help target niche markets and in the case of 18:4n-3 rich canola, have the potential to bypass the rate-limiting step in the FA desaturation pathway allowing for increased bioconversion to 20:5n-3 and 22:6n-3. Together these properties dictate high quality oil, which would serve as a suitable substitute for FO.

Table 2.3 Fatty acid composition of several vegetable and marine oils (RT – rainbow trout; AS – Atlantic salmon)

Study	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	20:5n-3	22:6n-3	SFA	MUFA	PUFA
Canola (Rapeseed)										
Przybylski, 2005	3.8	1.7	58.2	20.1	9.6	na	na	6.2	64.2	29.6
Bell *et al.,* 2003b	7.2	1.9	48.3	17.9	8.9	na	na	11.4	55.6	32.6
Jordal *et al.,* 2005	5.7	1.7	53.6	19.5	8.6	na	na	9.1	61.1	30.1
Camelina						na	na			
Putnam *et al.,* 1993	7.80	2.96	16.77	23.08	31.20	na	na	na	na	na
Rokka *et al.,* 2002	12.90	5.24	21.73	22.76	33.32	na	na	na	na	na
Abramovič & Abram, 2005	6.43	2.57	17.40	16.90	35.20	na	na	na	na	na
Flax (Linseed)										
Putnam et al., 1993	5.12	4.56	24.27	16.25	45.12	na	na	na	na	na
Przybylski, 2005	5.3	3.3	17.9	14.7	58.7	na	na	9.0	18.1	72.9
Bell *et al.,* 2003b	7.6	3.6	16.6	13.6	45.4	na	na	13.2	34.6	96.7
Collins *et al.,* 2011	5.0	3.4	16.2	16.3	58.3	na	na	8.7	16.5	74.8
Soybean										
Grisdale-Helland *et al.,* 2002	12.7	3.7	20.3	45.4	5.3	na	na	18.2	25.4	56.3
Przybylski, 2005	6.0	4.0	16.5	72.4	0.5	na	na	11.2	16.7	72.1
Marine (Wild)										
Blanchet *et al.,* 2005 (AS)	na	na	na	1.2	0.5	6.6	13.1	19.0	53.7	27.3
Blanchet *et al.,* 2005 (RT)	na	na	na	4.2	1.7	8.1	32.2	24.4	17.0	58.6
Marine (Farmed)	na	na	na							
Blanchet *et al.,* 2005 (AS)	na	na	na	7.4	1.6	7.9	15.2	25.6	33.4	41.0
Blanchet *et al.,* 2005 (RT)	na	na	na	6.2	1.0	7.3	18.7	26.9	32.5	40.6

Table 2.4 Effect of replacing fish oil with vegetable oils on the DHA and EPA contents of rainbow trout and Atlantic salmon. Values are % of total lipid

Study	DHA+EPA FO-fed fish	Fish oil replacement used in test group	DHA+EPA test group
Rainbow trout			
Caballero *et al.,* 2002	6.9	20 % FO + 80 % canola/palm	3.1
Bell and Dick, 2004b	5.3	Linseed/Sunflower	2.4
Rinchard *et al.,* 2006	9.2	Linseed	1.9
Drew *et al.,* 2007	21.2	Linseed/Canola	4.4
Collins *et al.,* 2011	14.5	Linseed	8.9
Atlantic salmon-freshwater			
Ng *et al.,* 2007	22.0	Palm	8.3
Bell *et al.,* 2001	20.6	Canola	8.3
Tocher *et al.,* 2002	28.1	Linseed	6.4
Brandsen *et al.,* 2003	23.8	Sunflower	10.8
Grisdale-Helland *et al.,* 2002	15.0	Soybean	9.2
Østbye *et al.,* 2011	48.7	Rapeseed	43.2
Atlantic salmon- seawater			
Bell *et al.,* 2003a	21.7	Canola	10.4
Bell *et al.,* 2003b	18.4	Linseed	5.8
Bell *et al.,* 2004a	12.4	Linseed	4.4
Berge *et al.,* 2009	14.4	Soybean	5.7
Rørå *et al.,* 2005	21.0	Soybean	4.5
Atlantic cod			
Morais *et al.,* 2012	17.4	Camelina	10.8

Camelina is another potential FO alternative that is also a member of the *Brassicaceae* family. It is known by a variety of names including: false flax, Dutch flax, gold of pleasure and linseed dodder. Originating in the Mediterranean to Central Asia, (Putnam *et al*., 1993; Plessers, 1962) camelina has been mainly known as a weed co-existing with flax. It quickly became utilised for its meal and oil due to the crop's hardy characteristics and short growing season (Fröhlich and Rice, 2005). The oil is used in both industrial and edible products such as cosmetics, nutraceuticals, pharmaceuticals and food spread (Putnam *et al*., 1993; Eidhin *et al*.,

2003), however there is gaining interest for its use as a biofuel. Camelina oil contains high levels of n-3 FA and is low in saturated FA. Putnam *et al*. (1993) reported camelina oil containing roughly 12 % saturated FA, 54 % PUFA, primarily linoleic and linolenic and 24 % MUFA, primarily oleic and eicosenoic acid. Camelina appears to be similar in FA content to flax, however camelina contains more sulphur (Putnam *et al*., 1993). As with other VO, camelina oil contains bioactive compounds γ-tocopherols (400-480 ppm) and sterols including: brassicasterol (133 ppm), campesterol (893 ppm), stigmasterol (103 ppm), Δ^5-avenasterol (393 ppm) and β-sitosterol (1,884 ppm) which act as antioxidants allowing for increased shelf life and stability (Shukla *et al.,* 2002). The most unusual characteristic of camelina oil is its relatively high content of cholesterol of 188 ppm or about 5% (Shukla *et al.,* 2002). Most VO contain less than 1 % cholesterol, whereas tropical oils contain relatively large amounts.

Flax has been grown since the beginning of civilisation and can be used for a variety of different purposes including: fibre production, oil, medicine, paper and linoleum (Przybylski, 2005). The most common flax variety grown in Canada is brown seeded flax, however it does come in a yellow variety. Brown flax is high in PUFA and is therefore a great source of n-3 FA, especially 18:3n-3 (>50 %) and is low in both saturated and MUFA, roughly 9 and 18 % respectively (Przybylski, 2005). Flax oil contains the least amount of natural antioxidants when compared to other VO with only 200-ppm γ-tocopherol, 20-ppm α-tocopherol, 120-ppm plastochromanol-8 and very little Δ-tocopherol. This is one of the main reasons why flax oil oxidises quicker than canola and camelina oils, the other is its high PUFA content (Bell *et al.,* 2001). Due to its high degree of oxidation great care must be taken when storing this oil to ensure optimal quality. The sterol content of flax oil is similar to that of other VO at approximately 50 % β-sitosterol, 25 % campesterol, 10 % Δ^5-avenasterol, 8 % stigmasterol, and

≤1 % brassicasterol (Przybylski, 2005). Bell *et al*. (2001) reported that flax oil inclusion should not exceed 50 % of supplementary lipid in Atlantic salmon diets. Inclusion levels above 50 % caused significant decreases in the n-3:n-6 PUFA ratio and the 20:5n-3 and 22:6n-3 concentrations such that the nutritional benefits to humans are considerably reduced.

2.8.3 *Alternative plant oils*

2.8.3.1 Stearidonic acid (SDA) rich oil

Stearidonic acid (18:4n-3) is formed by Δ6 desaturase and is the first metabolite in the n-3 FA conversion pathway of 18:3n-3 to 20:5n-3 and 22:6n-3 (Gunstone, 2011). As previously mentioned, the bioconversion of 18:3n-3 to 18:4n-3 *via* Δ6 desaturase is the rate limiting step in the n-3 biosynthetic pathway (Sprecher, 1981). Most commercial VO contain high levels of 18:2n-6 and 18:3n-3, which are present before this rate-limiting step in the FA pathway, however some plants such as *Echium plantagineum,* possess high levels of 18:4n-3. Feeding 18:4n-3 rich VO may be a feasible replacement for FO as it bypasses the rate-limiting step in the production of LC-PUFA. In support of this notion, human (Harris *et al.,* 2008) and dog (Harris *et al.,* 2007) studies have reported that the efficiency of conversion of 18:4n-3 to 20:5n-3 ranged from 17-30 % of the efficiency of feeding 20:5n-3 directly compared to less than 10 % for 18:3n-3. This suggests that bypassing Δ6 desaturase by feeding 18:4n-3 is a viable strategy for increasing the production of 20:5n-3 and 22:6n-3 in salmonids. Bharadwaj *et al.* (2010) reported a significantly higher muscle 22:6n-3 concentrations in hybrid striped bass fed 18:4n-3 compared to fish fed 18:3n-3. However, studies investigating the use of 18:4n-3 in salmonid diets have found mixed results. Miller *et al.,* (2007a) reported that feeding canola/echium oil-based diets containing 14 % 18:4n-3 to Atlantic salmon maintained the 20:5n-3 and 22:6n-3 levels in tissues. However

subsequent studies reported that feeding 18:4n-3-rich diets to Atlantic salmon and arctic char (*Salvelinus alpinus*) resulted in decreased levels of 20:5n-3 and 22:6n-3 (Tocher *et al*., 2006; Miller *et al*., 2007b).

2.8.3.2 Coriander oil

Coriander (*Coriandrum sativum* L.) is an annual herb belonging to the Apiaceae (*Umbelliferae*) family (Neffati and Marzouk, 2008). It is grown mainly in India, Asia and Europe but is also cultivated in North America. There are two types of coriander seed harvested in Saskatchewan, small and large seed. A large seed harvest will have an average yield of approximately 900 kg ha^{-1}, whereas a small seed harvest will yield slightly more (Government of Saskatchewan, 2008). This plant is cultivated mainly for its seeds, which have several properties in the use of cosmetics, food, drugs and herbicides, however the leaves are also widely used as an herb in Eastern and Indian cooking. Several studies report several benefits of coriander including, but not limited in, treatment of rheumatism, gastrointestinal upsets, insomnia, flatulence and joint pain (Wichtl, 1994; Delaquis *et al.,* 2002; Burt, 2004; Neffati and Marzouk, 2008). The chemical composition of the seed contains roughly 18 % oil depending on strain. Approximately 80% of coriander oil consists of an n-12 FA known as petroselinic acid (PA; 18:1n-12) (Reiter *et al*., 1998; Weber *et al*., 1995). 18:1n-12 is a positional isomer of 18:1n-9 with the double bond being in position 12 instead of 9 and can be split by oxidative cleavage into medium chain acids, C6 (adipic acid) and C12:0 (lauric acid) (IENICA, 2002). There is substantial evidence that there are health benefits associated with consuming fats with high levels of the monounsaturated FA, 18:1n-9 (Weber, *et al*., 1995; López-Huertas, 2009). However little is known about the effects of diets containing high levels of 18:1n-9 isomers such as 18:1n-12 and 18:1n-7 (vaccenic acid). Weber *et al*. (1995, 1997) demonstrated that ingestion of

coriander oil in rats led to incorporation of 18:ln-12 into heart, liver and blood lipids. Furthermore, a significant reduction in the concentration of 20:4n-6 was observed in the lipids of heart, liver and blood with a concomitant increase in the concentration of 18:2n-6 compared with results for the other groups. Weber *et al*. (1995) concluded that 18:ln-12 from dietary TAG is absorbed by rats as readily as 18:1n-9 and reduces the concentration of 20:4n-6 in tissue lipid. In a similar experiment Weber *et al*. (1999) similarly concluded that levels of 20:4n-6 were lowered when feeding diets containing coriander oil. The mechanism is still unclear, but the notion is that levels of 20:4n-6 decreased due to the presence of 18:ln-12, due to the Δ6 double bond that inhibits the Δ6 desaturase as a pseudo-product by mimicking the structure of 18:3n-6, as a precursor to 20:4n-6 (Weber *et al*., 1995, 1997, 1999). Furthermore, it was noted that DHA concentrations were lower in phosphatidylethanolamines and phosphatidylcholines of brain when coriander oil was fed even though all diets contained similar proportions of ALA. Therefore, Weber and associates (1999) speculate that dietary petroselinic acid may impair the formation of DHA by inhibition of desaturation/chain elongation reactions in the biosynthesis of n-3 LC-PUFA.

The seed oil also contains a variety of volatile compounds, include but are not limited to linalool, geranyl acetate and γ-terpinene (Delaquis *et al.,* 2002; Bhuiyan *et al.,* 2009). These volatile compounds, especially linalool, present in the seed oil, are believed to inhibit growth of a variety of microorganisms and have the potential to inhibit lipid peroxidation (Burt, 2004; Wangensteen, Samuelsen and Malterud, 2004; Silva *et al.,* 2011). Information on the mechanism of action has not been studied in great detail, but a review by Burt (2004) suggests that there is not one specific mechanism but several targets within the cell. Linalool, the primary volatile compound found in coriander oil can reach levels between 60-70 % (Guenther, *et al.,* 1950; Sriti

et al., 2010) and is mainly found in the form of its S (+) enantiomer. Silva *et al.* (2011) reported that linalool is known to cause increased permeability only in negatively charged membranes, supporting its antibacterial properties.

2.9 HYPOTHESES

Based on the previous observations, the levels of 20:5n-3 and 22:6n-3 in fish tissues may be increased by: 1) the use of VO with high concentrations of 18:3n-3, 2) the use of high quality oils with low levels of oxidation, 3) the addition of 18:1n-12, *via* coriander oil to the diet and 4) the use of VO containing 18:4n-3. Therefore I hypothesise that rainbow trout fed diets based on oils high in 18:3n-3 and 18:4n-3 with low oxidation levels and the addition of 18:1n-12 will significantly increase the levels of 20:5n-3 and 22:6n-3 in fish tissues.

3 EFFECT OF DIETARY PETROSELINIC ACID AND VEGETABLE OIL SOURCES ON FILLET FATTY ACID COMPOSITION OF RAINBOW TROUT

3.1 ABSTRACT

A 16-week feeding trial was conducted to examine the effect of adding petroselinic acid (PA; 18:1n-12), found in coriander oil, to vegetable oil (VO) diets on the bioconversion of linoleic acid (LA) to arachidonic acid (ARA; 20:4n-6) and α-linolenic acid (ALA; 18:3n-3) to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in rainbow trout. The experimental treatments were a 4 x 2 factorial arrangement of diets using four dietary oils (fish, flax, canola and camelina oils) and two levels of coriander oil (0 and 5 g kg^{-1} inclusion levels). Twenty-four tanks of triploid female rainbow trout (130 g initial weight; n = 3) were used in the experiment. Fish were fed to satiation twice per day. The fatty acid (FA) composition of fillets from coriander-fed fish had increased concentrations of 20:5n-3 and 22:6n-3 ($P < 0.05$). There was a trend to increased 20:5n-3 + 22:6n-3/20:4n-6 ratios when coriander oil was added to the diet ($P = 0.067$). These results suggest that the addition of 18:1n-12, *via* coriander oil, to VO diets can significantly increase the bioconversion of 18:3n-3 to 20:5n-3 and 22:6n-3 in rainbow trout.

3.2 INTRODUCTION

The health benefits of consuming n-3 long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are well established (as reviewed by Van Horn *et al.,* 2008) and the primary sources of these lipids in the human diet are through fish and seafood. It has been established that the fatty acid (FA)

composition of fish generally reflects that of the diet fed (Bell *et al.,* 2003b; Robin *et al.,* 2003; Izquierdo *et al*., 2003; Glencross, 2009; Thanuthong *et al.,* 2011), yet can differ between aquaculture raised and wild fish due to differences in nutrient composition (Kris-Etherton *et al.,* 2000). Salmonids in the wild, being higher in the food chain, primarily obtain 20:5n-3 and 22:6n-3 through direct consumption of phytoplankton, which produce them *de novo*, or indirectly through invertebrates and smaller fish (Jensen *et al.,* 2012), whereas those raised on farm generally obtain 20:5n-3 and 22:6n-3 primarily from marine fishmeal (FM) and fish oil (FO) (Bell *et al.,* 1994; Jensen *et al.,* 2012). However, limited supplies of FO require the development of alternative lipid sources (Miller *et al.,* 2011; Jensen *et al.,* 2012), with the most readily available alternative source being vegetable oils (VO). However, a main concern for the industry revolves around VO being relatively poor sources of n-3 FA and devoid of health beneficial 20:5n-3 and 22:6n-3 (Drew *et al.,* 2007; Miller *et al.,* 2011; Torstensen *et al*., 2011).

Traditionally, it has been known that biosynthesis of LC-PUFA from plant oils requires the desaturation and elongation of the 18-carbon precursors linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3; Tocher, 2003; Thanuthong *et al.,* 2011). However, in a review by Cunnane (2003), it is reported that rats deficient in LC-PUFA can synthesise 18:2n-6 and 18:3n-3 *in vivo via* hexadecadienoate (16:2n-6) and hexadecatrienoate (16:3n-3), respectively and/or tetradecadienoate (14:2n-6) and tertadecatrienoate (14:3n-3), respectively. Further research is needed to verify if this is the case in salmonids as well as to determine the efficiency of bioconversion of 16:3n-3 to 20:5n-3 and 22:6n-3 versus 18:3n-3. Nevertheless, it is presently known that salmonids are unable to synthesise 18:2n-6 or 18:3n-3 *de novo* and therefore these FA must be provided in the diet. However, they do possess the enzymes necessary for the bioconversion of 18:2n-6 to 20:4n-6 and 18:3n-3 to 20:5n-3 and 22:6n-3 (Tocher, 2003). The

first rate-limiting step in bioconversion of dietary 18:2n-6 and 18:3n-3 to their respective LC-PUFA is Δ6 desaturase (Vanger, 2011) and since both pathways use the same enzymes, competition is inevitable (Sprecher, 2002; Portolesi *et al*., 2007).

LC-PUFA also play an important metabolic role as precursors of eicosanoids and docosanoids (Reviewed by: Calder, 2009). Higher n-6:n-3 FA ratios in salmonid diets can lead to higher n-6:n-3 LC-PUFA ratios in subsequent fish tissue PL, which may be a risk factor for increased inflammatory response due to increased production of n-6 over n-3 derived eicosanoids (Bell *et al.,* 1993). Based on these observations, the ratio of dietary 18:2n-6:18:3n-3 is not only important for the FA composition of fish products, it also has a significant effect on lipid metabolism and inflammatory response in fish. Thus complete replacement of FO with VO should be performed with caution.

Petroselinic acid (PA; 18:1n-12) is a monounsaturated FA found in the *Umbelliferae* (coriander, parsley, fennel), *Araliaceae* and *Garryaceae* genera (Kleiman and Spencer, 1982; Cahoon *et al.*, 1992; Mekhedov *et al*., 2001), at levels up to 80 % of total FA (Fore *et al*., 1960; Kleiman and Spencer, 1982; Kiralan *et al*., 2009). Weber *et al.* (1995, 1997, 1999) observed that, in rats fed VO with similar or lower 18:2n-6, 18:1n-12 reduced the level of 20:4n-6 in heart, liver, and blood by what is thought to be the inhibition of Δ6 desaturase, whereby 18:1n-12 mimics the structure of 18:3n-6, a Δ6 desaturated product and precursor of 20:4n-6. Note that a 20:4n-6 reduction was not observed in brain total lipid of rat fed coriander oil but was observed in PL classes (Weber *et al.,* 1999). Based on these observations, we hypothesise that addition of 18:1n-12 to diets fed to rainbow trout (*Oncorhynchus mykiss*) will decrease the production of 20:4n-6 in the fillet and increase the bioconversion of 18:3n-3 to 20:5n-3 and 22:6n-3 resulting in the improved FA composition of trout fillets and balance of n-6:n-3 eicosanoids.

3.3 MATERIALS AND METHODS

3.3.1 *Oil preparation*

The flax seed (CDC Bethune; McDougall Acres Ltd., Moose Jaw, SK), canola seed (Bayer Crop Science, Saskatoon, SK), Camelina seed (Terramax Corporation, Qu'Appelle, SK) and coriander seed (Diefenbaker Seed Processors, Elbow, SK), were cold pressed at the bioprocessing pilot plant (University of Saskatchewan) using a continuous screw Komet oil press (type CA 59 G3; IBG Monforts Oekotec GmbH & Co. KG, Germany) with a 6 mm choke and operating with a screw speed of 70 rpm for flax seed and 87 rpm for canola, Camelina and coriander seed. Human grade menhaden FO was obtained from Bioriginal Ltd., Saskatoon, SK. To prevent oxidation of the oils, vitamin E (Lutavit® E 50; BASF Corporation, Florham Park, NJ) and butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO) were added the oils at levels of 5 g kg^{-1} oil and 4 g kg^{-1} oil respectively and N_2 gas was bubbled through the oils during production. Oils were stored under N_2 at 4 °C until used.

3.3.2 *Diets*

Diets were formulated to be iso-nitrogenous, iso-energetic and iso-lipid and were based on digestible nutrient values. The ingredient and FA composition of the diets are shown in Tables 3.1 and 3.2. Diets differed only in oil sources and were formulated to contain 17.6 MJ kg^{-1} digestible energy, 136.8 g kg^{-1} crude fat and 386.4 g kg^{-1} of digestible CP. The diets met or exceeded all other nutrient requirements of rainbow trout (NRC, 2011). The diets used in the feeding experiment were mixed in a Legacy Hobart Floor Mixer (Hobart Corporation, Troy, OH) for 15 minutes and then cold extruded in a 3 mm 4822 Hobart Food Grinder (Hobart Corporation, Troy, OH). Following extrusion, the diets were dried in a forced air oven (55 °C, 12

hours), chopped and screened to obtain a uniform pellet size, approximately 10 mm in length.

3.3.3 *Fish husbandry*

Fish used in the experiment were maintained in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2005). Triploid rainbow trout (initial weight 130 g) were purchased from Wild West Steelhead (Lucky Lake, SK) and maintained in 150 L tanks that were part of a semi-closed recirculation system (two water changes per hour). Water temperature was maintained at 15 ± 2.5 °C and dissolved oxygen (8.3 mg L^{-1}), pH (7.5) and water temperature were observed and recorded daily. Chlorine (0.0 mg L^{-1}), nitrate (10.8 mg L^{-1}), nitrite (0.1 mg L^{-1}) and ammonia (0.1 mg L^{-1}) were monitored on a weekly basis. Photoperiod was maintained at 14 h light/10 h dark cycle using incandescent lighting at 7 lux.

The 16-week experiment was a completely randomised design with a 4 x 2 factorial arrangement of treatments: four oils (fish, flax, canola and camelina) and two levels of coriander oil (0 and 5 g kg^{-1} inclusion levels). The experiment used 24 tanks of sixteen rainbow trout with three replicates per treatment. At the end of the experiment, 3 fish per tank were killed by a hard blow to the head, fillets excised, vacuum-packaged and stored at -20 °C until analysis.

3.3.4 *Analytical methods*

Proximate analysis was used to determine the moisture (AOAC, 1990; method no. 934.01), energy (oxygen bomb calorimetry; Parr Adiabatic Calorimeter, Model 1200), lipid (acid ether extract; AOAC, 1995; method no. 954.02), ash (AOAC, 1990; method no. 924.05) and protein content of all four of the experimental diets. The nitrogen content of samples was obtained using a combustion nitrogen analyser (Leco FP-528, St. Joseph, MI; AOAC 1995, method no. 990.03). Crude protein (CP) was estimated by multiplying nitrogen content by 6.25.

Oxidative stability index (OSI; AOCS, 1998; method cd 12b-92) was analysed by SunWest Food Laboratory Ltd. (Saskatoon, SK, Canada). Each sample was prepared for analysis by vacuum distillation at an airflow of 9 ± 0.2 L $hour^{-1}$ and 100 °C in a Metrohm Rancimat apparatus (Mississauga, ON; Model 679).

Diets and fish fillets were analysed for their FA profile. Whole fish fillets were removed from skin, fins and bones, cut into pieces no larger than 1 cm^2 and thoroughly combined and uniformly ground using a food processor (Moulinex DPA2, France) in the frozen state. Diets were ground using a ZM 100 Retsch Mill fitted with a 1 mm screen (Retsch GmbH, Haan, Germany). Direct FA methylation was then performed according to the procedure described by O'Fallon *et al*. (2007), with minor differences: the samples were vortex-mixed using a single tube vortex instead of a multi-tube vortex, and samples were centrifuged (Beckman Coulter J6-MC Centrifuge, Mississauga, ON) for 5 minutes at 1500 rpm. Non-methylated C13:0 (Sigma-Aldrich, Inc., St. Louis, MO) was used as the internal standard, and all other chemicals used were of GC grade, and obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Several crystals of *Tert*-butylhydroquinone, 97 % (TBHQ) (Sigma-Aldrich, Inc., St. Louis, MO, USA), were added to the tube to prevent lipid oxidation.

The main FA analysed in this study were: 18:1n-9, 18:3n-3, 18:2n-6, 20:4n-6, 20:5n-3, 22:6n-3 and 18:1n-12. Carcass and diet FA composition was determined by gas chromatography using an Agilent 6890 system and integrated with Agilent ChemStation software (Agilent Technologies, Mississauga, ON) and identified by use of external standards (Supelco 37 fatty acid methyl ester (FAME) mix; Cat# 47885). Pressurised helium, air and hydrogen were purchased from Praxair Canada Inc. (Mississauga, ON). FA methyl esters were separated using the following GC program: FA methyl esters were separated on a 30 m SGE BPX 70 column

with an initial oven temperature of 70 °C and held for 1 minute. The temperature program was 30 °C/min, 170 °C (0 min); 10 °C/min, 220 °C (0 min); 3 °C/min, 250 °C (5 min) for a total run time of 24:33 minutes. The machine was set for a 0.5 µl injection, split at a ratio of 75:1. The injector set points were a temperature of 250 °C, pressure of 22.12 psi and a total flow of 80.7 mL min^{-1}. Identification of 18:1n-12 was performed by comparison to its external standard. Separation of 18:1n-12 from 18:1n-9 was clear for all VO diets except for canola, where levels of 18:1n-9 are higher causing overlap of these peaks. Run time was increased to 40 minutes for these samples to better separate the peaks. The concentrations of individual FA were expressed as area % of total FAME. All results are based on duplicate analyses.

3.3.5 *Statistical analysis*

Data were analysed using a completely randomised design with a 4 x 2 factorial arrangement of treatments using the General Linear Model of PASW (IBM, Chicago, USA, V. 19.0, 2011). Differences between VO were determined using the Ryan–Einot–Gabriel–Welsch-F test. Treatment effects were considered significant when $P < 0.05$.

3.4 RESULTS

Overall, there were no significant differences or negative effects seen for growth, specific growth rate (SGR), average daily gain (ADG), average daily feed intake (ADFI) or feed conversion ratio (FCR) between dietary treatments, however ADFI showed a significant interaction between oil source and coriander addition ($P < 0.01$, Table 3.3). Fish fed diets containing flax and coriander oil consumed more feed on a daily bases, as indicated by increased FCR, compared to fish fed the remaining 7 treatments.

Table 3.1 Ingredient and proximate composition of the diets (g kg^{-1} dry diet)

Ingredients	Fish	Fish^{+C}	Flax	Flax^{+C}	Canola	Canola^{+C}	Camelina	Camelina^{+C}
Oil source	120.4	120.4	120.4	120.4	120.4	120.4	120.4	120.4
Coriander oil	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0
Blood meal	32.04	31.0	32.04	31.0	32.04	31.0	32.04	31.0
Poultry meal	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Pea protein concentrate	243.1	240	243.1	240	243.1	240	243.1	240
Canola protein concentrate	250.0	250.0	250.0	250.0	250.0	250.0	250.0	250.0
Corn gluten meal	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Celite	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Dicalcium Phosphate	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
DL - Methionine	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Choline Chloride	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Aqua Vitamin & mineral[a]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Proximate composition of the experimental diets (% dry matter)								
Dry matter	97.20	96.47	97.33	97.68	97.53	97.69	97.53	97.76
Acid ether extract	18.81	20.48	18.67	20.45	18.54	19.82	17.91	16.41
Crude protein	46.33	45.65	46.64	45.47	46.56	43.85	46.41	44.57
Ash	13.84	13.76	14.00	13.7 6	13.67	13.81	14.30	13.84
Digestible energy (MJ kg^{-1})	21.83	22.12	21.89	22.03	22.06	21.98	21.88	22.15

$^{+C}$Coriander oil was added to four of the experimental diets at 0.5 % of the total the diet; [a]As reported previously (Randall and Drew, 2010)

Table 3.2 Fatty acid compositions (% of total fatty acids) and oxidative stability indices of the experimental diets

Diet	18:1n-12	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3	OSI[1] (h)
Canola	nd	51.9	21.5	10.5	0.6	nd	nd	35.6
Canola^{+C}	2.1	50.2	21.6	10.4	0.3	nd	nd	23.9
Flax	nd	24.0	20.2	40.7	0.3	nd	nd	6.2
Flax^{+C}	4.7	24.7	19.6	36.1	1.5	nd	nd	2.7
Camelina	nd	28.3	28.0	26.8	2.1	nd	nd	20.8
Camelina^{+C}	5.5	26.9	26.3	23.4	1.9	nd	nd	14.5
Fish	nd	28.8	17.1	4.5	2.9	20.8	9.7	8.1
Fish^{+C}	5.6	30.4	16.4	3.3	3.3	15.6	8.0	5.7

[1]Oxidative stability index; $^{+C}$Addition of coriander oil; nd = Not detected

Table 3.3 Growth performance of fish fed diets containing canola, flax, camelina or fish oil with and without coriander oil

Oil Source	***SGR***[1] ***(% d^{-1})***	***ADG***[2] ***(g d^{-1})***	***ADFI***[3] ***(g d^{-1})***	***FCR***[4] ***(g feed g gain^{-1})***	***Initial Weight (g)***	***Final Weight (g)***
Canola	0.56	1.05	1.30	1.26	134.93	252.92
Flax	0.55	0.99	1.60	1.61	128.63	239.33
Camelina	0.50	0.96	1.28	1.46	139.47	247.27
Fish	0.66	1.30	1.48	1.15	131.83	277.82
Pooled S.E.M.[5]	0.05	0.12	0.35	0.06	3.63	14.84
Coriander						
Control	0.57	1.13	1.44	1.32	137.93	264.63
Coriander	0.58	1.06	1.39	1.42	129.50	244.04
Pooled S.E.M.	0.04	0.09	0.25	0.07	2.56	10.49
P-values						
Oil	0.176	0.234	0.178	0.063	0.224	0.323
Coriander	0.773	0.395	0.661	0.382	0.033	0.184
Interaction	0.588	0.537	0.006	0.323	0.322	0.423

[1]SGR, specific growth rate = [ln final weight – ln initial weight] / time (days) x 100
[2]ADG, Average daily gain = final weight - initial weight / number of days on trial
[3]ADFI, Average daily feed intake = total daily feed intake / number of days on trial
[4]FCR, Feed conversion ratio = total feed intake / average daily gain
[5]S.E.M., Standard error of the mean

The OSI of oils in all the diets were greater than 2.7 h indicating the oils were not highly oxidised (Table 3.4). However, addition of coriander oil to the diets lowered the OSI of the oils in the pelleted feeds, which may suggest reduced oil stability for all four oils. All of the VO diets were completely devoid of 20:5n-3 and 22:6n-3 but did contain small amounts of 20:4n-6 (Table 3.2). In contrast, the FO-containing diet, contained 20.8 % 20:5n-3 and 9.7 % 22:6n-3, however when coriander oil was added it reduced these concentrations to 15.6 and 8.0 %. Fish fillet FA composition, mimicked that of the oils fed (Table 3.4), as found with many other studies (Turchini *et al.,* 2009). Flax and camelina oil fed fish had the highest levels of 18:3n-3 (25.2 %) and 18:2n-6 (21.9 %) respectively, and FO-fed fish had significantly lower levels of these FA. The levels of 20:5n-3 and 22:6n-3, in fillets from fish fed the VO diets were significantly lower ($P < 0.05$) than in the fillets from fish fed the FO diet. Fish fed coriander oil had significantly

increased levels of 20:5n-3 and 22:6n-3 ($P < 0.01$). The concentration of 20:4n-6 in the fillet was numerically but not significantly reduced for fish fed coriander oil ($P = 0.285$). An interaction was found for 20:5n-3 in the fillet between oil source and coriander addition. Fish fed VO had increased concentrations of 20:5n-3 when coriander oil was present in the diet, while fish receiving FO diets had no changes in 20:5n-3 levels even when coriander oil was present. The 20:5n-3 + 22:6n-3 to 20:4n-6 ratio shows an increasing trend when coriander oil was added to the diet ($P = 0.067$).

3.5 DISCUSSION

Replacement of FO with VO generally causes a decrease in the tissue levels of 20:5n-3 and 22:6n-3 (as reviewed by Glencross 2009). This is due mainly to the low efficiency of the LC-PUFA biosynthetic pathway in salmonids (as reviewed by Turchini *et al.,* 2009). Bell *et al.* (2001, 2003a, 2003b) fed Atlantic salmon canola oil-based diets and obtained results similar to those reported in this experiment for 20:5n-3 and 22:6n-3. However, several strategies have been used to increase the production of LC-PUFA in VO-fed fish. Washout is one such strategy where fish are switched from a VO-based diet to a FO-based diet during a short period of time in the finishing phase of the production cycle. However, while some studies have found that this strategy brings 20:5n-3 and 22:6n-3 levels back to those found in fish fed FO diets throughout the growing period (Bell et al., 2003b; Bell et al, 2004a; Torstensen *et al*., 2005), other studies have reported reduced levels of 20:5n-3 and 22:6n-3 (Regost *et al.*, 2001; Regost *et al*., 2003a; 2003b) compared to controls. Furthermore, this method does not eliminate reliance on FO. The addition of coriander oil decreased the OSI of all 4 oils suggesting that 18:1n-12 might decrease the oxidative stability of other PUFA.

Table 3.4 Effect of oil source and addition of coriander oil on the fatty acid composition of fish fillets from rainbow trout. Values are % of total fat

Main effects	**Fatty Acid**							
Oil	**18:1n-12[1]**	**18:1n-9**	**18:2n-6**	**18:3n-3**	**20:4n-6**	**20:5n-3**	**22:6n-3**	**20:5n-3+22:6n-3 / 20:4n-6**
Canola	4.5^{ab}	44.3^{c}	18.6^{b}	5.7^{a}	0.5^{a}	2.8^{a}	9.1^{a}	8.0^{b}
Flax	3.7^{a}	24.7^{a}	17.3^{b}	25.2^{c}	3.3^{b}	2.8^{a}	6.7^{a}	3.5^{a}
Camelina	4.5^{ab}	28.3^{a}	21.9^{c}	14.7^{b}	3.6^{b}	3.2^{a}	8.6^{a}	3.4^{a}
Fish	5.1^{b}	26.7^{a}	13.9^{a}	2.8^{a}	1.5^{a}	10.8^{b}	20.5^{b}	22.0^{c}
Pooled S.E.M.[2]	0.30	1.69	0.76	1.11	0.38	0.25	1.12	1.46
Coriander								
Control	0.0	29.2	18.2	14.5	2.7	5.0^{a}	11.5^{a}	8.6
Coriander	4.6	27.5	17.0	10.8	2.1	5.9^{b}	12.1^{b}	11.1
Pooled S.E.M.	0.21	1.19	0.54	0.79	0.27	0.17	0.79	1.03
P-values								
Oil	nd	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Coriander	nd	0.522	0.418	0.586	0.285	< 0.01	0.005	0.067
Interaction	nd	0.185	0.403	0.801	0.839	0.004	0.157	0.368

[1]Petroselinic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic and docosapentaenoic acid

[2]Standard error of the mean; [a-c]Means with different superscripts are significantly different ($P < 0.05$); nd = Not done

Flax oil contains between 35-53 % 18:3n-3 (Bell *et al.,* 2003a, 2003b; Eidhin *et al.,* 2003), suggesting that flax-oil fed fish should have higher tissue LC-PUFA levels than fish fed canola oil. However, previous studies have reported that the levels of 20:5n-3 and 22:6n-3 in rainbow trout (Collins *et al.,* 2011) and Atlantic salmon (Bell *et al*., 2003a, 2003b) fed flax oil are similar to those of fish fed canola oil. This agrees with the results of the present study. The flax oil-fed fish had 20:5n-3 and 22:6n-3 similar to levels reported in other studies and these levels did not significantly differ from the tissue 20:5n-3 and 22:6n-3 levels in the canola oil-fed fish.

This suggests that the concentration of 18:3n-3 in canola and flax oils is not the limiting factor in the synthesis of 20:5n-3 and 22:6n-3. Bell *et al.* (2010) reported no effect on growth when feeding camelina oil to Atlantic salmon smolts and liver 18:3n-3 and its elongation product 20:3n-3 increased while all other n-3 FA decreased when FO was replaced with a VO blend of rapeseed, palm and camelina in a 5:3:2 ratio.

Petroselinic acid (18:1n-12), the major FA found in coriander oil, is a positional isomer of octadecenoic acid with its double bond being in position 6 instead of 9. This makes separation between these FA and *cis*-vaccenic acid difficult. Since canola oil diets contain high levels of octadecenoic acid the 18:1n-12 values are masked resulting in lower 18:1n-12 values. Coriander oil contains approximately 80 % 18:1n-12 (Fore *et al.*, 1960; Mallet *et al.*, 1990; Weber *et al.* 1995; Reiter *et al*., 1998; Kiralan *et al.*, 2009).

Weber *et al.,* (1995; 1999) reported that the addition of 63 g kg^{-1} 18:1n-12 inhibited desaturase and elongation reactions of 18:2n-6 in liver lipids and reduced 20:4n-6 in the lipids of heart, liver and blood by almost 50 % ($P < 0.01$) in the rats. They suggested that 18:1n-12 might lead to a decrease of 20:4n-6 in tissue lipids by inducing pseudo-product mediated inhibition of

Δ6 desaturase. In the present study, addition of coriander oil at 5 g kg^{-1} of the oil caused a significant increase in 20:5n-3 and 22:6n-3 and a trend to increased 20:5n-3 + 22:6n-3/20:4n-6 concentration (P = 0.005 and 0.067 respectively). However, while 20:4n-6 concentrations in fillets decreased by approximately 25 %, it was not significant. Since, Δ6 desaturase is found in both the n-3 and n-6 FA pathways, it is possible that this effect may be due to difference in binding site receptors of the Δ6 desaturases within the FA pathways, however further research is needed to verify this notion.

The results of the present study suggest that the supplementation of coriander oil to commercial VO diets may be an effective way to increase n-3 LC-PUFA and decrease n-6 LC-PUFA, however more research needs to be performed in order to determine the most optimal level of inclusion. Furthermore, in order to decrease the possibility of coriander oils pungent odour transferring to fish flesh the oil would need to be deodorised to remove these volatile compounds. Taken together the results of this study suggest that the addition of 18:1n-12 to VO diets may result in an increase in the levels of 20:5n-3 and 22:6n-3 in fish products and reduce aquaculture's dependence on FO.

4 EFFECTS OF DIETARY SUPPLEMENTATION OF PETROSELINIC ACID (18:1N-12), IN CANOLA OIL DIETS ON THE METABOLSIM OF [1-^{14}C] 18:3N-3 AND [1-^{14}C] 18:2N-6 IN RAINBOW TROUT HEPATOCYTES

4.1 ABSTRACT

The overall aim of this study was to investigate the effects of petroselinic acid (PA, 18:1n-12), found in coriander oil, on the ability of rainbow trout hepatocytes to increase the production of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) from [1-^{14}C] α-linolenic acid (ALA; 18:3n-3) and to reduce the production of arachidonic acid (ARA; 20:4n-6) from [1-^{14}C] linoleic acid (LA; 18:2n-6).

Seventy-two rainbow trout (200 ± 0.8 g) were allocated to 12 tanks (3 replicates) and fed one of four canola oil based diets containing no fishmeal (FM) and/or fish oil (FO) and varied inclusions of coriander oil (0, 0.5, 1.0 and 1.5 %) for two weeks. Isolated hepatocytes from each group were then incubated with [1-^{14}C] 18:3n-3 and [1-^{14}C] 18:2n-6 in order to study how coriander oil influences their metabolism.

[1-^{14}C] 18:2n-6, 18:3n-3 substrates and their fatty acid (FA) products were primarily esterified in the PL fraction of the hepatocytes, with the highest percentages at the 0.5 % coriander oil inclusion level. Esterification into triacylglycerols (TAG), pooled mono- and diacylglycerols and cholesterol esters were lowest in the 0.5 % inclusion levels for both substrates with only 1-2 % of the total radioactivity from both substrates recovered as free FA (FFA). The main metabolites recovered from [1-^{14}C] 18:3n-3 and [1-^{14}C] 18:2n-6 in all treatment levels was the unchanged substrate, 20:5n-3, 20:3n-6 and 20:4n-6 respectively. Recovery into 20:5n-3 was highest in the 0.5 % and 1.5 % inclusion levels of coriander oil. Increased production of 22:6n-3 was seen at the 0.5 and 1.0 % inclusion levels, however a slight decrease

was noticed at the 1.5 % inclusion level.

β-oxidation, on the other hand, was highest at the 1.5 % inclusion of coriander oil in both substrates. Acetate, a main breakdown product of FA *via* peroxisomal β-oxidation, decreased three-fold in [1-^{14}C] 18:2n-6 and nearly doubled for [1-^{14}C] 18:3n-3 when coriander was added at increasing levels. Acyl Coenzyme A oxidase enzyme activity (ACO) showed no significant differences between treatments. Relative gene expression of Δ6 desaturase decreased with 0.5 % addition of coriander oil compared to the control. Overall, the addition of 18:1n-12 *via* coriander oil to VO based diets containing no FM or FO, significantly increased anti-inflammatory precursor 22:6n-3 ($P = 0.011$) and decreased pro-inflammatory precursor 20:4n-6 ($P = 0.023$) in rainbow trout hepatocytes.

4.2 INTRODUCTION

The desirability of fish in human diets combined with increasing populations has increased the demand for fish products. However, fish production from capture fisheries has plateaued at approximately 95 million tonnes and most fisheries are fully exploited (FAO 2009; Gillund and Myhr, 2010). This drop in fish production has raised the demand for aquaculture-derived products and aquafeed production, resulting in increased demand of the primary ingredients for salmonid diets: fish oil (FO) and fishmeal (FM). As wild fish stocks continue to decline and demand increases, FO and FM need to be replaced with alternative ingredients.

Vegetable oils (VO) have become the primary alternative for FO as they contain high levels of essential C18 fatty acid (FA). Flax and camelina oils are rich in α-linolenic acid (ALA; 18:3n-3) and canola oils is rich in oleic acid (OA; 18:1n-9), making these VO preferred over corn and sunflower oil, which are high in linoleic acids (LA; 18:2n-6). 18:3n-3 and 18:2n-6 are the FA precursors to bio-active, long-chain polyunsaturated fatty acids (LC-PUFA)

eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6), which are precursors for both anti- and pro-inflammatory eicosanoids, respectively. Diets containing elevated levels of n-6 FA can cause an imbalance in n-3:n-6 ratio, in humans (Simopoulos, 2008) and in Atlantic salmon (Bell *et al.,* 1991), causing increased production of pro-inflammatory eicosanoids *via* 20:4n-6, which plays a pivotal role in many inflammatory related conditions and diseases (Simopoulos, 2008). Numerous studies have investigated the differences between using blends of these VO and use on their own, however no matter the combination these VO do not account for the loss of LC-PUFA in the form of 20:5n-3 and 22:6n-3 (Bell *et al.,* 2001; Torstensen *et al.,* 2005; Turchini and Francis, 2009; Pettersson *et al.,* 2009). In order for the aquaculture industry to sustain the use of these alternative VO sources, a method, by which to reduce the highly inflammatory product 20:4n-6 and increase the health beneficial products 20:5n-3 and 22:6n-3, needs to be determined.

Coriander (*Coriandrum sativum*) is a member of the Umbelliferae family and is grown all over the world primarily as a spice. Its ground seeds and oil have been used for treatment of rheumatism, gastrointestinal upsets, insomnia, flatulence and joint pain in humans (Wichtl, 1994; Said *et al.,* 1996; Emamghoreishi *et al.,* 2005), has been shown to positively influence lipid profile in plasma of rats when diets contained cholesterol (Ramadan *et al.,* 2008) and its oil has been shown to have anti-microbial and anti-inflammatory properties (Reuter *et al.,* 2008). To the best of our knowledge, coriander oil has not been used in fish diets. Petroselinic acid (18:1n-12), the major FA found in coriander oil, is a positional isomer of octadecenoic acid with its double bond being in position 6 instead of 9 (Fore *et al.*, 1960; Mallet *et al.*, 1990; Weber *et al.* 1995; Reiter *et al*., 1998; Kiralan *et al.*, 2009). 18:1n-12 has been detected at level between 68-83 % in coriander oil (Kiralan *et al.*, 2009; Fore *et al.*, 1960; Weber *et al.* 1995; Mallet *et al.*, 1990;

Reiter *et al*., 1998) and has been shown to aid in the reduction of 20:4n-6 in heart and liver of rats (Weber *et al*., 1995; 1997), have antimicrobial properties (Charvet *et al*., 1991) and can be a competitive inhibitor of topoisomerases, therefore having potential in treatment of cancer (Suzuki *et al.,* 2000). With regard to the reduction of 20:4n-6, 18:1n-12 has been shown to strongly inhibit desaturation and chain elongation reactions of 18:2n-6 (Weber *et al.,* 1997) and therefore may have potential uses in the aquaculture industry due to the increase in feeding of plant products. Weber and associates (1995; 1997) report that 18:1n-12 may modulate FA composition in cell membrane PL leading to a decrease in inflammatory precursor 20:4n-6 by acts of inducing pseudo-product mediated inhibition of Δ6 desaturase. We hypothesise that the addition of 18:1n-12, *via* coriander oil, to canola oil-based diets will decrease the production of 20:4n-6 and increase the production of 20:5n-3 and 22:6n-3 in rainbow trout (*Oncorhynchus mykiss*) hepatocytes when fed diets containing no FM and FO.

4.3 MATERIALS AND METHODS

4.3.1 *Chemicals and Reagents*

The radiolabelled FA [1-^{14}C] 18:3n-3 and [1-^{14}C] 18:2n-6 (specific radioactivity 50 mCi mmol^{-1}) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). The non labelled 18:3n-3 and 18:2n-6, essential FA free bovine serum albumin (BSA), foetal bovine serum (FBS), Leibovitz-15 (L-15), 20,70 dichlorfluorescein, 20,70- dichlorfluorescein and collagenase, Phosphate buffer saline (PBS), phenylethylamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), BHT from Sigma-Aldrich (St. Louis, MO, USA), sodium bicarbonate solution, L-glutamine, were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and metacain MS-222 from Norsk Medisinaldepot (Norway). Ammonium dihydrogenphosphate, perchloric acid ($HClO_4$), Thin-

layer chromatography (TLC)- plates, all solvents and other chemicals for FA and coriander analysis were purchased from Merck (Darmstadt, Germany). Vitamin E was purchased from BASF Corporation (Florham Park, NJ, USA) and the BHT from Sigma-Aldrich (St. Louis, MO, USA). Coriander seed (*Coriandrum sativum*) was a gracious gift from Diefenbaker Seed Processors Ltd. (Saskatchewan, Canada). FA peaks were identified by comparison with the standard mixture GLC-68 A (Nu-check Prep, Inc, Elysian, Minnesota, USA).

4.3.2 *Oil preparation*

Coriander was cold pressed at the bioprocessing pilot plant (University of Saskatchewan) using a continuous screw Komet oil press (type CA 59 G3; IBG Monforts Oekotec GmbH & Co. KG, Germany) with a 6 mm choke and operating with a screw speed of 87 rmp. Antioxidants were added during processing in the form of vitamin E (Lutavit® E 50) and BHT at levels of 5 g kg^{-1} oil and 4 g kg^{-1} oil respectively and were aerated with nitrogen to reduce oxidation.

4.3.3 *Diet preparation*

The diets were produced at The Centre of Feed Technology at the University of Life Sciences (Ås, Norway). The experimental diets are VO based (canola oil) with four coriander oil inclusion levels of 0, 0.5, 1.0 and 1.5 %, yielding three VO diets containing coriander oil and one basal diet containing no coriander oil.

All diets were formulated to be iso-nitrogenous, iso-energetic and iso-lipidic contain no FM and/or FO and contained 40.3 % crude protein (CP), 19.1 % total lipid and a digestible energy value of 17.9 MJ $kcal^{-1}$. The ingredient and FA composition of the diets is shown in Tables 4.1 and 4.2. The diets used in the feeding experiment were mixed for 15 minutes, and cold pelleted in an Italgi Pressa P35A pasta machine (Carasco, Italy) fitted with a 3 mm die.

Following pelleting, the diets were dried in a forced air oven (65 °C, 8 hours) and screened to obtain a uniform pellet size, approximately 8 mm in length.

Table 4.1 Ingredient and proximate composition of the diets (g kg^{-1})

	Diets			
Coriander oil inclusion	0 %	0.5 %	1.0 %	1.5 %
Ingredients				
Canola oil	150.0	145.0	140.0	135.0
Coriander oil	0.0	5.0	10.0	15.0
Canola protein concentrate	300.0	300.0	300.0	300.0
Pea protein concentrate	150.0	150.0	150.0	150.0
Soy protein concentrate	24.0	24.0	24.0	24.0
Corn gluten meal	255.9	255.9	255.9	255.9
Wheat flour	80.0	80.0	80.0	80.0
Monocalcium phosphate	34.6	34.6	34.6	34.6
Vitamin/Mineral premix[a]	5.0	5.0	5.0	5.0
Vitamin C	0.5	0.5	0.5	0.5
Proximate composition of the experimental diets (% dry matter)				
Dry matter	95.38	94.54	93.09	95.62
Crude lipid	19.29	18.24	18.86	19.22
Crude protein	70.44	67.49	69.76	70.74
Ash	7.27	7.90	7.62	7.52
Digestible Energy (MJ kg^{-1})	23.33	23.04	23.35	23.42

[a]Rovimix Stay C-35; New Jersey, USA

4.3.1 *Fish Husbandry*

Seventy-two rainbow trout with a mean initial weight of approximately 200 ± 0.8 g (Aquaculture Protein Centre, Ås, Norway) were housed in 200 L fiberglass tanks with a 12 h light and 12 h dark cycle. They were divided into twelve tanks (three tanks per treatment with 6 fish per tank) and fed one of four diets based on four coriander oil inclusions (0, 0.5, 1.0 or 1.5 %) for two weeks. All tanks were supplied with freshwater at a rate of 8 l min^{-1} and a temperature of 8 ºC ± 2.

Table 4.2 Fatty acid composition of the diets (% of total fatty acids), (n = 3)

	Diet			
Fatty acids	**0 %**	**0.5 %**	**1.0 %**	**1.5 %**
Saturates				
14:0	0.13	0.16	0.14	0.13
16:0	9.30	9.61	9.68	8.85
18:0	2.92	2.93	2.92	2.70
20:0	0.97	1.19	0.97	1.05
22:0	0.52	0.52	0.53	0.51
24:0	0.27	0.25	0.26	0.24
∑SAT[1]	18.25	19.05	18.73	16.93
Monoenes				
16:1[2]	0.26	0.25	0.25	0.27
18:1[3]	59.74	57.19	58.99	63.01
20:1 n-11	0.29	0.50	0.16	0.30
20:1 n-9	1.19	1.15	1.13	1.27
22:1[4]	0.50	0.46	0.48	0.46
24:1 n-9	0.11	0.11	0.11	0.11
∑MUFA[5]	62.16	59.75	61.21	65.46
Polyunsaturates				
18:2 n-6	4.56	4.52	4.52	4.27
18:3 n-6	0.06	0.05	0.04	0.00
20:2 n-6	1.95	2.02	1.96	1.43
20:3 n-6	nd	nd	nd	nd
20:4 n-6	nd	nd	nd	nd
18:3 n-3	0.68	0.74	0.78	1.48
20:3 n-3	nd	nd	nd	nd
20:4n-3	0.43	0.28	nd	0.18
20:5 n-3	0.62	0.89	0.85	0.32
22:5 n-3	nd	0.12	nd	nd
22:6 n-3	nd	nd	nd	nd
∑PUFA[6]	8.29	8.62	8.15	7.66
Other	7.98	8.54	12.61	9.23

nd = not detected; [1]Includes 15:0, 17:0; [2]Includes 16:1n-7, 16:1n-9, 16:1n-10; [3]Includes 18:1n-7, 18:1n-9, 18:1n-12; [4]Includes 22:1n-7, 22:1n-9, 22:1n-11; [5]Includes 14:1n-5, 15:1, 17:1n-7

Dissolved oxygen was measured daily. Four diets were randomly allocated to triplicate tanks and feed was distributed by hand twice daily until apparent satiation (08:00 and 15:00). There were no mortalities during the feeding trial.

4.3.2 *Proximate analysis*

Ingredients and experimental diets were ground using a ZM 100 Retsch Mill (Retsch GmbH, Haan, Germany) with a 1 mm screen. Analysis of all samples were conducted in duplicate using the following methods, moisture content (Association of Official Analytical Chemists (AOAC) 1995; method no. 930.15); ash by incineration (AOAC 1990; method no. 942.05); CP was reported as 6.25 times total nitrogen and was determined using a micro-Kjeldahl (AOAC 1990; method no. 984.13) with the following modifications: samples were digested with 15 mL H_2SO_4, 3.5 g K_2SO_4 and 0.4 g $CuSO_4$, then boiled for 45 min at 420 °C, after cooling, 25 mL is dissolved with distilled water; raw fat, Accelerated Solvent Extraction (Technical Note 68).

4.3.3 *Fat Extraction and Total Fatty Acid Analysis*

Plasma lipids were extracted from cell suspension by use of the Bligh and Dyer method (1959). FA were derivatised and analysed as methyl esters using capillary gas chromatography on a HP 6890 equipped with a BPX-70 column (60 m x 0.25 mm x 0.25 μm) (SGE Analytical Science Pty Ltd, Ringwood, Australia). The temperature program initiated at 70 °C for 1 min, increased by 30 °C/min to 170 °C, 1.5 °C/min to 200 °C and 3 °C/min to 220 °C with a final hold time of 5 minutes. Peaks were integrated with HP GC ChemStation software (rev. A.05.02) (Agilent Technologies, Little Falls, DE), and identified by use of external standards. Coefficients of variation were < 5 %. The concentrations of individual FA were expressed in % of total FA. All results are based on duplicate analyses.

4.3.4 *Isomer Verification*

18:1n-12 isomer verification was performed as follows: One sample was split injected (20:1) into an Agilent 7890A gas chromatograph interfaced with a 5975C mass selective detector (Agilent Technologies, Little Falls, DE). The FAME were separated on an HP-5MS fused silica capillary column (50 m x 0.25 mm x 0.25 μm) (Agilent Technologies, Little Falls, DE) using helium as carrier gas at an average velocity of 26 cm sec^{-1} (0.5 mL min^{-1}). The initial temperature was 30 °C for 3 min, then increased by 10 °C/min to 200 °C, then by 5°C/min to 240 °C, and then by 4 °C/min to 300 °C and kept for 20 min. MS ion source temperature was 200 °C, with electron ionisation energy of 70 eV. MS acquisition was recorded both in the TIC and SIM mode. Identification of compound was performed using an EI spectrum of standard compounds (Sigma Aldrich, St. Louis, MO) and specific fragment ions were used for quantification.

4.3.5 *Isolation of Hepatocytes*

At the end of the 14 day feed period, hepatocytes were isolated from one fish per tank (3 fish per diet). The fish were anaesthetised in MS-222 and the livers were perfused following the two-step collagenase procedure developed by Seglen (1976) and modified by Dannevig and Berg (1985) and conducted as per Kjær *et al.* (2008). The hepatocytes were seeded at the density of 10 million cells per cell flask (25 cm^2), one for each radiolabelled substrate and one for gene expression, and incubated at 12 ºC for 24 h before addition of FA. Cells for RNA isolation, FA and Acyl Coenzyme A oxidase (ACO) analysis were stored at -80 ºC.

4.3.6 *Incubation of Hepatocytes with Radiolabelled Fatty Acids*

The isolated hepatocytes were incubated with 35 nmol, 2.5 μCi of [1-^{14}C] 18:3n-3 and [1-^{14}C] 18:2n-6, a final FA concentration of 7 μM, in 5 mL of GlutaMAX culture medium containing: 0.01 M HEPES (Sigma-Aldrich), 9 mM Sodium biocarbonate (Sigma-Aldrich) and 10 mL L^{-1} Penicillin-Streptomycin (Invitrogen) without FBS. The radiolabelled FA were added to the medium as potassium salts bound to BSA at a ratio of 2.7:1 mol. The cells were incubated at 12 ºC for 24 h.

4.3.7 *Lipid Analysis in Cells and Medium*

The hepatocytes were washed once in 1 % albumin in PBS and twice in PBS before harvesting in PBS. Lipids from cells and medium incubated with [1-^{14}C] 18:3n-3 and [1-^{14}C] 18:2n-6 were extracted according to Folch *et al.* (1957). The chloroform extract was divided into several parts for measurement of: total radioactivity (not methylated), FA analysis (HPLC) and lipid class analysis (TLC). The extracted radiolabelled lipid was then methylated according to Mason and Waller (1964) and FA composition of the cells was determined by reversed phase high-pressure liquid chromatography as described by Narce *et al.* (1988). The mobile phase was acetonitrile:water (85:15 v/v) at a flow rate of 1 mL min^{-1} and a temperature of 30 °C. The column used was a symmetry 3.5 μm C_{18} column and the FA were detected with a radioactive detector A-100 (Radiomatic Instrument & Chemicals, Tampa, FL, USA). The FA were identified by comparison of their retention times with those of external standards.

The lipids extracted from the cells and were then separated on TLC-plates into various classes using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:1 v/v/v). The separated lipid classes, phospholipids (PL), monoacylglycerides and diacylglycerides (MAG+DAG) free fatty acids (FFA), triacylglycerols (TAG) and cholesterol esters (CL) were

visualised by dipping the plate in a solution of 3 % cupric acetate in 8 % phosphoric acid and charred or 5 minutes at 140 ºC on a plate heater (Camag, Switzerland). Quantitative analysis of separated lipid classes, was completed by scanning the plates with a CAMAG TLC Scanner 3 (Camag, Switzerland). This was performed at a speed of 20 mm sec^{-1} and a data resolution of 100 µm $step^{-1}$ with a slit dimension of 6.00 x 0.45 mm at a wavelength of 400 nm. Lipid classes were identified by comparison to standards under UV-light. The lipid classes were scraped off and dissolved in scintillation fluid for scintillation counting (TRI-CARB 1900 TR, Packard Instruments).

4.3.8 *Measurements of radioactive β-oxidation products*

The capacity of *β*-oxidation was measured by determination of the ^{14}C-containing oxidation products, acid soluble products (ASP) and CO_2 as described by Christiansen *et al.* (1976). Measurements of radioactive CO_2 were performed by transferring 1.5 mL of the medium into 25 mL Erlenmeyer flasks stoppered with a rubber cap equipped with a suspended central well containing a Whatman filter paper with 0.3 mL phenylethylamine/methanol (1:1 v/v). The medium was acidified with 0.3 mL 1 M $HClO_4$ and ^{14}C-CO_2 was trapped for 1 h. Then the filter papers were placed in vials and dissolved in 8 mL of scintillation fluid for scintillation counting (Radiomatic Instrument & Chemicals, Tampa, FL, USA). The amount of ^{14}C-ASP was determined by adding 0.5 mL ice cold 2 M $HClO_4$ to the incubation medium and incubated at 4 ºC for 1 h, then the samples were centrifuged at 16,000 × *g* for 10 min at 4 ºC and 200 µl of the supernatant was collected for scintillation counting (Radiomatic Instrument & Chemicals, Tampa, FL, USA). The remaining supernatant was neutralised with NaOH and the different ASP were detected by using high-pressure liquid chromatography equipped with a ChromSep Inertsil C8-3 column (250 × 4.6 mm stainless steel), an UV-detector at 210 nm and radioactive detector

A-100 (Radiomatic Instrument & Chemicals, Tampa, FL, USA) coupled to the UV detector. The mobile phase was 0.1 M ammonium dihydrogenphosphate adjusted with phosphoric acid to pH 2.5, the flow rate was 1 mL min^{-1}. The components were identified by comparison to external standards and retention times.

4.3.9 *Determination of Protein Level*

To calculate the total protein concentration of the hepatocyte homogenates, a Total Protein kit was used following the protein precipitation method of Lowry/Peterson (Sigma, USA) (Lowry *et al.,* 1951; Peterson, 1977). The BSA standards were prepared by diluting 400 μg mL^{-1} of BSA in water. NaCl (final concentration of 0.1 M) was added to eliminate ampholyte interference. First, 0.15 % deoxycholate solution was added to both standard and sample. After 10 min incubation at room temperature, trichloroacetic acid (TCA) solution was added to precipitate protein. The samples were centrifuged and then the pellet was dissolved in Lowy reagent solution. After 20 min incubation, the Folin & Ciocalteu's phenol reagent working solution was added to every sample. The absorbance was read after 30 min incubation using a Titertek Multiskan® PLUS MKII plate reader (Labsystems, Finland).

4.3.10 *β-Oxidation: Acyl Coenzyme A oxidase (ACO) Assay*

Triplicate samples from each dietary group were analysed for ACO enzyme activity by homogenising isolated hepatocytes in 6x volume of homogenisation buffer (0.25 M sucrose (Sigma-Aldrich), 15 mM HEPES (Sigma-Aldrich), 1.0 mM EDTA (Sigma-Aldrich), 1.0 mM EGTA (Sigma-Aldrich), pH 7.4). The samples were then diluted 1:1 in 0.4 % Triton X-100 (Merck), centrifuged for 15 minutes at 4 °C and 800 *x* g. ACO activity and protein concentration were measured in the supernatant. The ACO assay was performed at 25 °C with an assay mix

consisting of 0.10 M TRIS (Sigma-Aldrich), pH 8.5, 0.05 mg mL^{-1} peroxidase (Sigma-Aldrich), 0.59 mg mL^{-1} BSA (Sigma-Aldrich), 0.01 mg mL^{-1} FAD (Sigma-Aldrich), 0.02 mg mL^{-1} 2'7'-dichlorofluorescin diacetate (Sigma-Aldrich), and 0.06 mg mL^{-1} PalmCoA (Sigma-Aldrich). Assay mix without PalmCoA was used as blank. The samples were recorded at 502 nm measuring absorbance chance on SPECTROstar^{nano} Microplatereader (BMG LABTECH).

4.3.11 *Extraction of Total RNA, cDNA Synthesis and Real Time PCR / Gene Expression*

An RNeasy® Mini Kit with on-column Rnase free DNase set (Qiagen, MD, USA) was used to isolate total RNA from the hepatocytes. All procedures were followed according to the manufacturer's instructions. RNA was eluted in RNase-free water (Eppendorf, Hamburg, Germany) and stored at -80 ºC. Purity and concentrations were measured by optical density (NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delware, USA).

cDNA was synthesised following a modified protocol from the AffinityScript QPCR cDNA synthesis Kit (Agilent Technologies, Stratagene, Ca, USA). A mix of oligo d(T) and random hexamers were used to prime the synthesis. The reaction was performed by incubating the samples at: 25 ºC for 5 minutes, 42 ºC for 5 minutes, 48 ºC for 40 minutes, and 95 ºC for 5 minutes. Primers for Real-Time PCR analysis (Table 4.3) were designed based on available rainbow trout sequences in the GenBank® using the Vector NTI Advance 11.5 (Invitrogen, Carlsbad, USA) and ordered from Invitrogen (Carlsbad, USA). Real-Time PCR for the dose response study was performed in a LightCycler480 (Roche Applied Science, Mannheim, Germany) with gene-specific primers for Δ6 desaturase. The PCR reaction mix consisted of 5 μL SYBR Green I Master mix (Roche Applied Science, Mannheim, Germany), 1 μL forward and

reverse primer (final concentration of 0.5 μM), and 4 μL 1:10 dilution of cDNA. All samples were analysed in duplicate with non-template control for each primer pair. Elongation factor 1a (EF1A) and eukaryotic translation initiation factor 3 (ETIF3) were used as reference genes. The PCR conditions were 95 ºC for 5 min, 45 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. Melting curve analysis (95 ºC for 5 sec and 65 ºC for 1 min, 97 ºC) was run to confirm the presence of a single PCR product. Primer efficiency was calculated for each primer pair (Table 4.6). The relative gene expression levels were calculated using the ΔΔCt method. Selection of EF1A as the most stable reference gene was performed with geNorm (Vandesompele *et al.,* 2002).

4.3.1 *Statistical analysis*

Linear and quadratic regression procedure of PASW (IBM, Chicago, USA, V. 19.0, 2011) was used to analyse the results. Quadratic regression parameters were removed, as none of them were significant. The Ryan-Einot-Gabriel-Welsh F test was used to determine differences between means. Differences in gene expression levels and ACO oxidase activity between the control group and the other dietary groups were assessed using a one-way AVOVA. Results were considered significant when $P < 0.05$.

Table 4.3 Primers used for real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.	Primer efficiency
Δ6 desaturase	agtggctcctctggtccgtg	ccgacaacatcaatgatggctt	AF301910	1.91
EF1A[1]	tccagcggcaaggtca	actccgcaggatgtagggct	AF498320	1.96
ETIF3[2]	caggatgttgttgctggatggg	acccaactgggcaggtcaaga	DW542195	1.97

[1]EFIA, Elongation factor 1a
[2]ETIF3, Eukaryotic translation initiation factor 3

4.4 RESULTS

4.4.1 *Diet fatty acid composition*

The FA compositions of the experimental diets are shown in Table 4.2. 18:1n-12 was not separated from its isomers due to difficulty in separation and was therefore included in the total MUFA, which does increase with increasing inclusion of coriander oil. The diets were formulated to change only in the amount of coriander oil added. However, FA analysis portrayed a major decrease in 18:2n-6 and 18:3n-3 when coriander oil was present in the diet. These diets were re-analysed, using gas chromatography, for FA composition at a different laboratory (SunWest Food Laboratory Ltd., Saskatoon, SK) to ensure accuracy. The results showed the same FA profiles for each diet, illustrating the possibility of coriander oil having an effect on the FA extraction process, as this was not an issue for the diet that did not contain coriander oil. This irregularity in FA composition when coriander oil is present in VO-based diets was also seen in a subsequent study (See section 5). In addition, the diets were also re-analysed using gas chromatography-mass spectromotery. The

4.4.2 *Hepatocyte fatty acid composition*

Table 4.4 shows the FA composition of the total lipid fraction of hepatocytes. 18:1n-12 was not detected in hepatocytes. Increasing inclusion of coriander oil caused a significant linear increase in 14:0 from 0.7 to 1.2 % ($P = 0.037$; Table 4.5), total saturated FA from 23 to 28 % ($P = 0.018$) (Table 4.5) and 20:5n-3 from 5.3 to 7.0 % ($P = 0.042$; Table 4.5). Furthermore, a significant linear decrease was seen for 18:1n-9 (Table 4.5) from approximately 19 to 14 % ($P = 0.044$) and total monounsaturated FA was reduced from 24 to 19 % ($P = 0.037$; Table 4.5). A linear trend for increased total 20:5n-3 + 22:6n-3 ($P = 0.066$; Table 4.5) was also seen with increasing inclusion of coriander oil.

Table 4.4 Fatty acid composition of the total lipid fraction (% of total fatty acids) of hepatocytes, (n = 3)

	Hepatocytes			
Coriander oil	0 %	0.5 %	1.0 %	1.5 %
Saturates				
14:0	0.7 ± 0.07	0.9 ± 0.04	0.8 ± 0.10	1.2 ± 0.31
16:0	16.7 ± 0.17	18.3 ± 0.46	17.1 ± 0.55	19.7 ± 1.65
18:0	5.5 ± 0.95	6.7 ± 0.24	6.9 ± 0.24	6.8 ± 0.59
24:0	0.1 ± 0.06	0.1 ± 0.01	0.3 ± 0.08	0.1 ± 0.03
∑SAT[1]	23.0 ± 1.44	26.3 ± 0.34	25.3 ± 0.85	28.0 ± 1.30
Monenes				
16:1n-7	0.1 ± 0.03	0.1 ± 0.01	0.3 ± 0.24	0.1 ± 0.03
18:1n-7	1.4 ± 0.69	2.0 ± 0.04	1.9 ± 0.01	2.0 ± 0.06
18:1n-9	18.7 ± 0.56	17.4 ± 1.07	17.4 ± 0.52	13.9 ± 2.67
18:1n-12	---	---	---	---
24:1n-9	0.9 ± 0.02	1.0 ± 0.05	1.0 ± 0.08	1.0 ± 0.13
∑MUFA[2]	24.3 ± 0.65	22.9 ± 1.11	22.6 ± 0.55	19.0 ± 2.82
Polyunsaturates				
18:2n-6	4.6 ± 2.24	0.2 ± 0.01	2.4 ± 2.22	0.2 ± 0.02
18:3n-6	0.9 ± 0.03	0.8 ± 0.11	0.8 ± 0.06	0.7 ± 0.05
20:2n-6	0.1 ± 0.0	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.02
20:4n-6	---	0.1 ± 0.03	1.1 ± 1.07	---
18:3n-3	0.0 ± 0.03	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.01
18:4n-3	0.5 ± 0.24	0.6 ± 0.10	0.7 ± 0.9	0.6 ± 0.09
20:3n-3	5.7 ± 0.98	5.1 ± 0.41	5.4 ± 0.20	4.7 ± 0.88
20:5n-3	5.3 ± 0.06	6.4 ± 0.08	5.4 ± 0.10	7.0 ± 0.44
22:5n-3	1.1 ± 0.57	2.1 ± 0.12	1.7 ± 0.13	1.9 ± 0.07
22:6n-3	31.7 ± 0.92	34.1 ± 1.40	33.0 ± 1.98	36.7 ± 2.28
∑PUFA	50.1 ± 1.46	50.1 ± 1.01	51.3 ± 0.52	52.3 ± 1.62
EPA+DHA	37.0 ± 0.97	40.5 ± 1.31	38.4 ± 1.98	43.6 ± 2.72

[1]Includes 20:0, 22:0; [2]Includes 20:1, 22:1; --- = Not detected

Table 4.5 Linear regression of the FA composition in the total lipid fraction (% of total FA) of hepatocytes, (n = 3)

Regression parameters	b_0	b_1	r^2	*P*-value
14:0	0.522	0.212	0.367	0.037
16:0	16.757	0.790	0.259	0.091
18:0	5.867	0.388	0.194	0.152
24:0	0.106	0.016	0.048	0.493
∑SAT[1]	23.532	1.409	0.443	0.018
16:1n-7	0.092	0.044	0.062	0.436
18:1n-7	1.533	0.184	0.139	0.232
18:1n-9	19.036	-1.460	0.347	0.044
24:1n-9	0.717	0.119	0.199	0.147
∑MUFA[2]	24.586	-1.596	0.365	0.037
18:2n-6	79.503	0.562	0.018	0.162
20:2n-6	79.503	0.562	0.018	0.889
20:4n-6	79.503	0.562	0.018	0.692
18:3n-3	79.503	0.562	0.018	0.181
18:4n-3	0.521	0.055	0.076	0.386
20:3n-3	5.647	-0.269	0.081	0.369
20:5n-3	5.405	0.408	0.353	0.042
22:5n-3	1.417	0.181	0.134	0.242
22:6n-3	31.794	1.375	0.254	0.095
∑PUFA	49.769	0.784	0.198	0.147
20:5n-3 + 22:6n-3	37.202	1.782	0.299	0.066

[1]Includes 20:0, 22:0; [2]Includes 18:1n-12, 20:1, 22:1

4.4.3 *Recovery of [1-^{14}C] FA total lipid fraction of hepatocytes and β-oxidation products*

The percentage distribution of radioactivity from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 in total lipid fraction of hepatocytes into lipid classes and oxidation products is shown in Table 4.6. No significant differences were observed between the two [1-^{14}C] substrates in the recovery of radiolabeled cellular lipids for any experimental diet after the 24 h incubation. Radiolabelled 18:2n-6, 18:3n-3 and their products were primarily esterified into the PL fraction of the hepatocytes, where they constituted 77-85 % and 81-86 % of the total radioactivity respectively, with the highest percentages at the 0.5 % coriander inclusion level for both radiolabelled substrates (Table 4.6). Esterification into TAG, pooled MAG+DAG and CL fractions were

lowest in the 0.5 % inclusion levels for both substrates. Only about 1-2 % of the total radioactivity from both substrates was not esterified but recovered as FFA. The breakdown of radiolabelled substrates to the *β*-oxidation products ASP and CO_2 showed the same opposing trend for both substrates. Increasing inclusions of coriander oil to [1-^{14}C] 18:2n-6 caused a linear increase in ASP from 37-53 % (P = 0.039; Table 4.7) and decrease in CO_2 from 63-48 % (P = 0.039; Table 4.7), whereas [1-^{14}C] 18:3n-3 showed a significant quadratic increase in ASP from 55-62 % (P = 0.019; Table 4.7) and decrease in CO_2 from 46-38 % (P = 0.019; Table 4.7).

Acetate, one of the main breakdown products of FA *via* the process of peroxisomal *β*-oxidation, showed an almost two-fold increase in [1-^{14}C] 18:3n-3 hepatocytes (P = 0.028; Table 4.8), where as [1-^{14}C] 18:2n-6 hepatocytes showed significant decrease (P = 0.020; Table 4.8), when coriander was added at increasing levels.

4.4.4 *Elongation and desaturation of [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3*

Table 4.9 illustrates the percentage distribution of radioactivity from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 into their desaturation and elongation products in total lipid fraction of hepatocytes. The main metabolites recovered from [1-^{14}C] 18:2n-6 in all treatment levels was the unchanged substrate, 20:3n-6 and 20:4n-6. Production of 20:2n-6 showed a significant increase from 6.9 to 13.1 % (0 % and 1.0 % diets, respectively) (P = 0.047; Table 4.10), whereas increased inclusion of coriander oil significantly reduced the recovery of [1-^{14}C] 18:2n-6 to 20:4n-6 from 25 % to 15 % (Table 4.9; P = 0.023, Table 4.10). The main radiolabelled FA recovered in all treatments from the [1-^{14}C] 18:3n-3 was the unchanged substrate and 20:5n-3. Recovery into 20:5n-3 was highest in the 0.5 % and 1.5 % inclusion levels of coriander oil at approximately 29 and 26 % respectively, but was not significant (Table 4.9).

Table 4.6 Percentage distribution of radioactivity from [1-^{14}C] 18:2 and [1-^{14}C] 18:3 in total lipid fraction of hepatocytes into lipid classes and oxidation products[a]

	Inclusion of Coriander Oil			
	0 %	0.5 %	1.0 %	1.5 %
[1-^{14}C] 18:2n-6				
Cellular lipid (nmol mg protein^{-1})	6.5±1.49	6.4±1.53	5.1±0.73	5.9±1.39
Hepatocyte lipids (%)				
PL	77.1±2.38	85.5±2.33	80.3±3.65	80.7±1.15
MAG + DAG	4.8±1.39	3.1±0.79	3.6±0.66	5.1±1.39
FFA	1.7±0.21	1.5±0.40	2.0±0.45	2.1±0.43
TAG	15.7±0.79	9.6±1.21	13.5±2.46	11.4±0.97
CL	0.6±0.21	0.4±0.03	0.6±0.15	0.6±0.25
Total oxidation products (nmol mg protein^{-1})	0.05±0.00	0.04±0.00	0.06±0.03	0.07±0.02
Oxidation products (%)				
CO_2	62.7±0.76	62.2±4.62	60.8±5.19	47.5±5.08
ASP	37.3±0.76	37.8±4.62	39.2±5.19	52.5±5.08
[1-^{14}C] 18:3n-3				
Cellular lipid (nmol mg protein^{-1})	6.0±0.52	4.3±0.30	6.4±0.44	5.9±0.27
Hepatocyte lipids (%)				
PL	83.5±2.38	86.1±1.95	80.9±3.29	84.1±0.33
MAG + DAG	3.6±0.51	3.2±0.28	3.7±0.46	3.6±0.69
FFA	1.7±0.18	1.7±0.49	1.8±0.34	2.3±0.45
TAG	10.9±1.65	8.9±1.32	13.2±2.49	9.7±0.96
CL	0.3±0.05	0.2±0.05	0.3±0.08	0.3±0.05
Total oxidation products (nmol mg protein^{-1})	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.01
Oxidation products (%)				
CO_2	45.5±2.65	51.3±2.46	45.6±3.48	37.6±2.21
ASP	54.5±2.65	48.7±2.46	54.3±3.48	62.4±2.21

[a]Hepatocytes from all diets were incubated with 7μM [1-^{14}C] FA. Data are means ± S.E.M. (n=3). PL, phospholipids; MAG+DAG, monoacylglycerols + diacylglycerols; FFA, free fatty acids; TAG, triacylglycerols; CL, cholesterol esters; ASP, acid-soluble products.

Table 4.7 Linear regression of the percentage distribution of radioactivity from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 in the total lipid fraction of hepatocytes and oxidation products

Regression parameters	b_0	b_1	r^2	*P*-value
[1-^{14}C] 18:2n-6				
Lipid Classes				
PL	79.503	0.562	0.018	0.676
MAG +DAG	3.758	0.154	0.009	0.766
FFA	1.453	0.144	0.076	0.387
TAG	14.794	-0.888	0.101	0.315
CL	0.493	0.027	0.013	0.727
Oxidation Products				
ASP	23.932	4.707	0.361	0.039
CO2	70.068	-4.707	0.361	0.039
[1-^{14}C] 18:3n-3				
Lipid Classes				
PL	85.882	-0.343	0.011	0.747
MAG +DAG	3.035	0.074	0.012	0.731
FFA	0.730	0.179	0.115	0.281
TAG	10.233	0.067	0.001	0.937
CL	0.119	0.024	0.073	0.397
Oxidation Products				
ASP	35.979	2.928	0.276	0.079
CO2	64.021	-2.928	0.276	0.079

The equation is in the form $y = b_o + b_1x$; y= fatty acid;
b_o = intercept; b_1 = slope; x = concentration of coriander in the diet

Table 4.8 Percentage distribution and linear regression of acetate production from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 oxidation products

Diet	0 %	0.5 %	1.0 %	1.5 %
[1-^{14}C] 18:2n-6				
Acetate (%)	14.51±1.40	8.10±0.73	12.79±2.05	5.09±0.01
[1-^{14}C] 18:3n-3				
Acetate (%)	9.13±1.14	5.26±0.149	13.96±1.88	14.14±1.84
Regression parameters	b_0	b_1	r^2	*P*-value
[1-^{14}C] 18:2n-6				
Acetate	15.869	-2.468	0.472	0.020
[1-^{14}C] 18:3n-3				
Acetate	4.695	2.371	0.397	0.028

The equation is in the form $y = b_o + b_1x$; y= fatty acid; b_o = intercept; b_1 = slope; x = concentration of coriander in the diet

Increased production of 22:6n-3 was seen at the 0.5 and 1.0 % inclusion levels, however a slight decrease was noticed at the 1.5 % inclusion level from 15 to 13.8 %. Overall, a significant decrease was seen for 18:4n-3 ($P = 0.037$; Table 4.10) and 22:6n-3 significantly increased from 8 to 15 % ($P = 0.011$; Table 4.10).

Table 4.9 Percent distribution of radioactivity from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 into their desaturation and elongation products in total lipid fraction of hepatocytes n=3

	Diets			
Coriander oil	0 %	0.5 %	1.0 %	1.5 %
1-^{14}C *18:2n-6*				
18:2n-6	44.8 ± 4.38	43.9 ± 4.45	48.4 ± 2.84	52.1± 3.15
18:3n-6	0.4 ± 0.41	1.0 ± 0.55	0.5 ± 0.03	2.0 ± 0.58
20:2n-6	6.9 ± 0.64	12.9 ± 1.33	13.1 ± 2.21	12.9 ± 1.81
20:3n-6	22.5 ± 2.23	23.9 ± 4.26	20.4 ± 1.29	16.7 ± 2.31
20:4n-6	25.0 ± 4.68	18.0 ± 1.67	17.2 ± 0.90	15.0 ± 1.55
1-^{14}C *18:3n-3*				
18:3n-3	58.9 ± 3.89	47.5 ± 4.15	55.1 ± 7.43	53.7 ± 2.09
18:4n-3	3.2 ± 0.43	2.6 ± 0.74	2.6 ± 0.63	1.4 ± 0.12
20:3n-3	---	---	---	---
20:4n-3	6.3 ± 2.19	7.6 ± 2.58	5.2 ± 2.01	3.5 ± 0.66
20:5n-3	22.0 ± 1.60	28.7 ± 0.49	25.3 ± 3.44	25.8 ± 0.41
22:6n-3	7.9 ± 0.13	11.7 ± 1.47	15.0 ± 1.49	13.8 ± 1.81
24:6n-3	1.7 ± 0.53	1.9 ± 0.47	2.0± 0.89	1.7 ± 0.10

[1]Includes 20:0, 22:0; [2]Includes 20:1, 22:1; --- = Not detected

4.4.5 *ACO Activity and Gene Expression*

No significant differences in the enzymatic activity of ACO oxidase between groups were found (Figure 4.1). The relative gene expression of Δ6 desaturase decreased with addition of coriander oil (Figure 4.2). Inclusion of 0.5 % coriander oil significantly decreased Δ6 desaturase gene expression compared to the 0 % control group, however further inclusion of coriander oil did not further reduce Δ6 desaturase gene expression.

Table 4.10 Linear regression of the distribution of radioactivity from [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 into their desaturation and elongation products in total lipid fraction of hepatocytes n=3

	b_o	b_1	r^2	*P*- value
[1-^{14}C] 18:2n-6				
Hepatocyte lipids (%)				
18:2n-6	43.32	5.31	0.225	0.119
18:3n-6	0.34	0.85	0.292	0.069
20:2n-6	8.76	3.59	0.338	0.047
20:3n-6	24.00	-4.19	0.245	0.102
20:4n-6	23.39	-6.09	0.418	0.023
DI 18:2n-6 (%)				
Linear	47.860	-4.635	0.998	0.001
[1-^{14}C] 18:3n-3				
Hepatocyte lipids (%)				
18:3n-3	54.95	-1.57	0.012	0.732
18:4n-3	3.27	-1.06	0.366	0.037
20:4n-3	7.29	-2.16	0.144	0.224
20:5n-3	23.71	0.58	0.004	0.852
24:6n-3	1.79	0.05	0.001	0.921
22:6n-3	8.98	4.17	0.496	0.011
DI 18:3n-3 (%)				
Linear	45.060	0.775	0.044	0.789

[a]Hepatocytes from all diets were incubated with 7µM [1-^{14}C] FA. The equation is in the form y = b_o + b_1x; y= fatty acid; b_o = intercept; b_1 = slope; x = concentration of coriander in the diet; DI= Desaturation index; $DI_{18:2n-6}$ (18:3 + 20:3 + 20:4n-6/18:2 + 18:3 + 20:2 + 20:3 + 20:4) x 100 and $DI_{18:3n-3}$ (18:4 + 20:4 + 20:5 + 22:5 + 22:6 + 24:6n-3)/18:3 + 18:4 + 20:4 + 20:5 + 22:5 + 22:6 + 24:6n:3) x 100. Values are significant when $P \leq 0.05$.

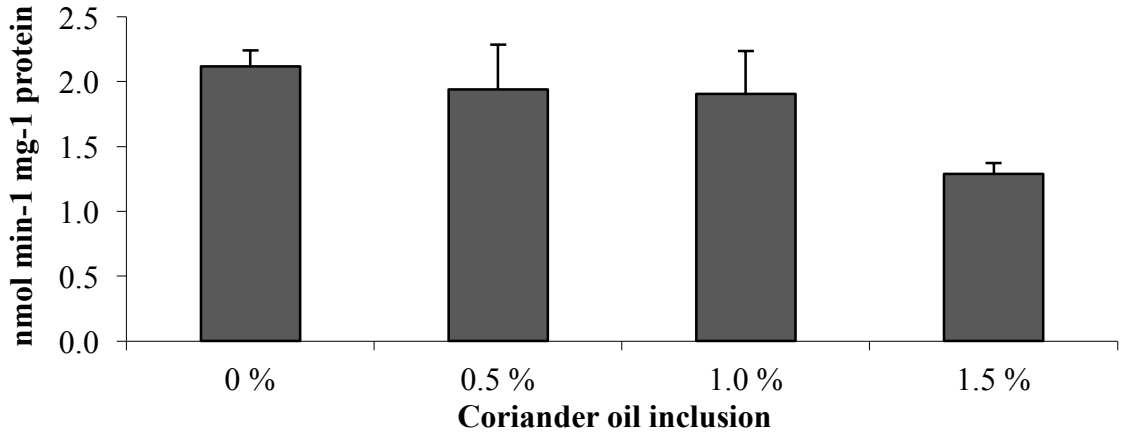

Figure 4.1 Relative ACO activity in hepatocytes isolated from rainbow trout of the four different dietary groups.

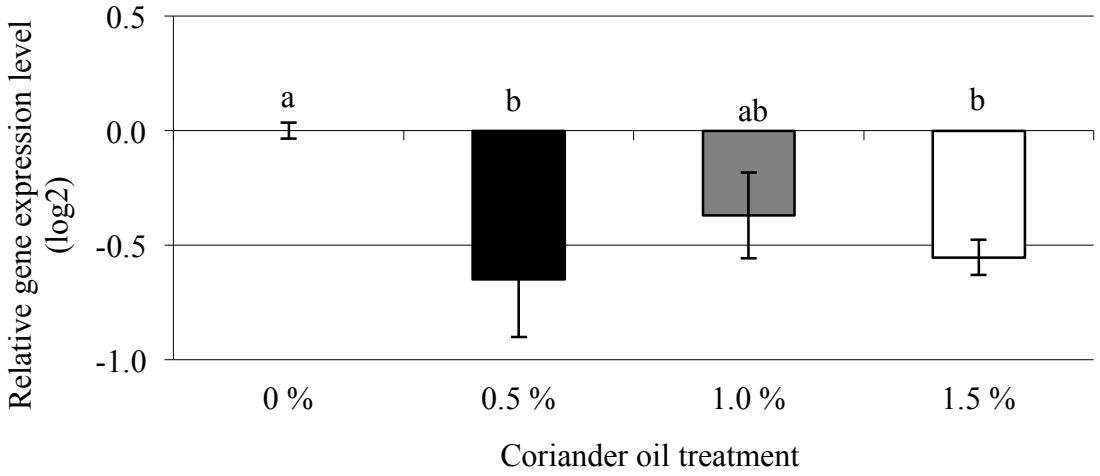

Figure 4.2 Relative gene expression level of Δ6 desaturase analysed with quantitative PCR in hepatocytes isolated from rainbow trout of the four different dietary groups. Data are presented as -ΔΔCt ± SE (n=6).

4.5 DISCUSSION

Addition of coriander oil to rainbow trout diets has, to our knowledge, only been examined in our lab. In a previous study, we found that addition of coriander oil to VO based diets resulted in increased levels of 20:5n-3 and 22:6n-3 in rainbow trout fillets (Chapter 3). Furthermore, the concentration of 20:4n-6 was numerically reduced when coriander oil was present in the diet, however it was not significant. Weber *et al.* (1995, 1997) demonstrated that ingestion of coriander oil in rats led to incorporation of 18:ln-12 into heart, liver and blood lipids. Moreover, a significant reduction in the concentration of 20:4n-6 was observed in the lipid of heart, liver and blood with a concomitant increase in the concentration of 18:2n-6 compared with results for the other treatment groups.

In the present study, addition of coriander oil to canola oil-based rainbow trout diets is an effective way to increase the production of 20:5n-3 and 22:6n-3 and reduce the production of 20:4n-6 in hepatocytes. The FA composition of hepatocyte lipid was influenced by addition of coriander oil. Increasing inclusion of coriander oil caused an increase in the percentages of 14:0,

total saturated FA and 20:5n-3 in hepatocyte lipids. These results are in contract with those of Moya-Falcón *et al.,* (2005) who found that rapeseed oil fed fish had significantly lower levels of these FA. Furthermore, the present study saw a reduction in 18:1n-9 and total monounsaturated FA, which also opposed the findings of Moya-Falcón *et al.,* (2005). LC-PUFA, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3 were very low or not present in the diets, however they were present in the hepatocyte lipid when compared to the control. Due to the duration of the feeding trial most of the endogenous FA available in the PL and TAG was probably present before the initiation of the trial, however even though the FA composition of the hepatocytes was not highly influenced this short term trial shows a change in the metabolism of the FA as indicated by the radiolabelled substrates as well as a tendency for increased levels of endogenous 20:5n-3 and 22:6n-3 and decreased level of 20:4n-6. This suggests that coriander oil has the potential to play a role in manipulating n-3 and n-6 FA metabolism in hepatocytes.

Two fates of FA after entering a cell are through *β*-oxidation or esterification into cellular lipids. Both long chain radiolabelled substrates were preferentially incorporated into PL with the highest percentages at the 0.5 % coriander inclusion level, with little radioactivity recovered in TAG. This coincides with reports from Moya-Falcón *et al.* (2004, 2005) who also found that PUFA were primarily incorporated into PL for long chain n-3 and n-6 PUFA and Vegusdal *et al.* (2004) who reported primary esterification into PL for [1-^{14}C] 18:1n-9 and [1-^{14}C] 20:5n-3 substrates. Radiolabelled FFA increased in response to increased levels of coriander oil for both substrates, indicating a possible pooling of un-metabolised FFA substrates.

Within the n-6 and n-3 FA pathways 18:2n-6 and 18:3n-3 can either be desaturated *via* Δ6 desaturase to 18:3n-6 and 18:4n3, respectively or elongated *via* Elovl 5 to 20:2n-6 and 20:3n-3, respectively (Guillou *et al.,* 2010). In the present study, response to increasing inclusion of

coriander oil caused, [1-^{14}C] 18:2n-6 to be elongated to its "dead-end" product 20:2n-6 more readily than following the direct passage through Δ6 desaturase as was expected. This may be an indication of competition for Δ6 desaturase between 18:2n-6 and 18:3n-3 (Sprecher, 1981; Kinsella *et al.* 1990). However, it must be noted that the percentage of 18:2n-6 in non-radiolabeled hepatocytes differed amongst treatments (Table 4.3), which may have influenced this increase.

When Atlantic salmon were fed diets containing a 75 % VO and 25 % FO (3.7:2:1, low erucic acid rapeseed:palm:LO) the conversion of [1-^{14}C] 18:2n-6 and [1-^{14}C] 18:3n-3 to "dead-end" products 20:2n-6 and 20:3n-3 was reduced (Stubhuag *et al.*, 2005). They suggested that the precise mechanism for these changes were not clear but may be the result of competitive effects with desaturases. In contrast, when Atlantic salmon were fed LO for 40 weeks at increasing levels Bell *et al.* (2004a) found increased levels of the "dead-end" products 20:2n-6 and 20:3n-3, when compared to the control. The increased elongation event of [1-^{14}C] 18:2n-6 to 20:2n-6 was followed by a subsequent decrease in both 20:3n-6 and 20:4n-6, suggesting that the theory reported by Weber *et al.* (1995; 1997) that 18:1n-12 is thought to act as a pseudo-mediated inhibitor of Δ6 desaturase causing a reduction in the inflammatory precursor 20:4n-6, (Weber *et al.,* 1997) may be accurate, however further research needs to verify this notion.

The bioconversion of [1-^{14}C] 18:3n-3 did, on the other hand, follow the direct passage through Δ6 desaturase as indicated by no presence of 20:3n-3 (Table 4.9). This was followed by a decrease in stearidonic acid (SDA; 18:4n-3) and a subsequent increase in 22:6n-3 *via* peroxisomal *β*-oxidation. The increase in 22:6n-3 and acetate from the [1-^{14}C] 18:3n-3 substrate could be an indication of increase peroxisomal *β*-oxidation. The Sprecher Pathway clearly defines that in order for 22:6n-3 to be produced it must pass through peroxisomal *β*-oxidation,

which produces acetate as a by-product (Sprecher, 2000). However, this is not in agreement with the results obtained for ACO activity, the rate-limiting step in peroxisomal *β*-oxidation (Small *et al.,* 1985), which showed no significant differences between treatments. Thanuthong *et al.* (2011) reported that there are discrepancies in whether or not *β*-oxidation activity is stimulated or depressed by dietary FA composition. Nevertheless, this potential increase in peroxisomal *β*-oxidation may occur at a different position within the pathway, which needs to be elucidated.

The relative expression of Δ6 desaturase, when compared to the control, was significantly down-regulated by coriander oil at the 0.5 % inclusion level. There is no definitive mechanism as of yet for why and how this decrease occurs, but the proposed mechanism previously suggested by Weber *et al.* (1995; 1997) may be correct. If this is the case, then there is potential for 18:1n-12 to reduce the expression of Δ6 desaturase in the n-6 pathway relative to the Δ6 desaturase in the n-3 pathway as seen by the reduction in 20:4n-6 and increase in 22:6n-3. Further research is needed to verify this theory as well as differentiate which Δ6 desaturase within the two pathways is being affected. In contrast, most studies where salmonids are fed VO based diets it is common to see increased expression of FA desaturase and elongase genes due to the reduced level of LC-PUFA (Zheng *et al.,* 2004; Miller *et al.,* 2008; Bell and Koppe, 2011).

4.6 CONCLUSION

Supplementing a canola oil based diet for rainbow trout with coriander oil changes the endogenous FA composition of hepatocytes, leading to increased 22:6n-3 as well as a reduction in 20:4n-6. It is difficult to draw conclusions about the actual process involved, therefore further research is needed to determine the mechanism in which coriander oil causes the increase in these health beneficial LC-PUFA.

5 FEEDING STEARIDONIC ACID (18:4N-3) ENRICHED LINSEED OIL WITH CORIANDER OIL INCREASES WHOLE BODY DHA CONCENTRATIONS IN RAINBOW TROUT

5.1 ABSTRACT

Rainbow trout have the ability to produce long-chain polyunsaturated fatty acids (LC-PUFA) when biosynthetic precursors are available. However, feeding stearidonic acid (SDA; 18:4n-3) instead of α-linolenic acid (ALA; 18:3n-3) bypasses Δ6 desaturase; the rate limiting step in n-3 biosynthetic pathway. This may result in the increased synthesis of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Female triploid rainbow trout (starting weight 42 g) were fed one of four vegetable oil (VO)-based diets containing either conventional linseed oil (LO) or 18:4n-3 enriched LO, supplemented with 0 or 15 g kg^{-1} coriander oil. Two dietary treatments containing fish oil, with 0 or 15 g kg^{-1} coriander oil were included as reference diets but not included in statistical analyses. Thus, the experiment was analysed as a completely randomised design with four dietary treatments. Fish were randomly assigned to 18 tanks (3 tanks per diet; 20 fish per tank). The fish were fed twice a day to apparent satiety for 75 days. At the end of the experiment, the whole-body fatty acid (WBFA) composition of the fish was determined using gas chromatography. There were no significant differences in the specific growth rate, average daily gain, average daily feed intake or feed conversion ratio of fish due to diet ($P > 0.05$). However, whole body 20:5n-3 and 22:6n-3 concentrations were significantly increased ($P < 0.05$) in fish fed SDA-enriched LO supplemented with coriander oil compared to fish fed conventional LO. Furthermore, the level of 20:4n-6 in fish fed the SDA-enriched LO diet supplemented with coriander oil was significantly higher than in the fish fed the SDA-enriched LO diet without added coriander oil which in turn,

was significantly higher than in fish fed either of the conventional LO diets. The results suggest that feeding 18:4n-3 with the addition of coriander oil may increase the WBFA concentrations of 20:4n-6, 20:5n-3 and 22:6n-3 in rainbow trout.

5.2 INTRODUCTION

The current scarcity of fish oil (FO) has necessitated the use of alternative oils in aquaculture diets. It is well established that simple replacement of FO with vegetable oils (VO) results in little to no effect on growth and health but causes decreased levels of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in fish tissues (Tocher *et al.,* 2003a; Bell *et al.,* 2004a; Glencross, 2009; Sanden *et al.,* 2011). Salmonids have the ability to convert the n-3 C_{18} PUFA precursor, α-linolenic acid (ALA; 18:3n-3) to LC-PUFA, 20:5n-3 and 22:6n-3, which has stimulated interest in the use of VO with high concentrations of 18:3n-3, such as LO as a FO substitute (Turchini *et al.,* 2011a, 2011b).

The first step in the step in the n-3 biosynthetic pathway is the conversion of 18:3n-3 to stearidonic acid (SDA; 18:4n-3) catalysed by Δ6 desaturase, which represents a bottleneck in the pathway. Thus, feeding 18:4n-3 may result in higher concentrations of 20:5n-3 and 22:6n-3 compared to feeding 18:3n-3. In support of this notion, in both human (James *et al.,* 2003; Harris *et al*., 2008) and dog (Harris *et al*., 2007) the efficiency of conversion of 18:4n-3 to 20:5n-3 ranged from 17-30 % of the efficiency of feeding 18:4n-3 directly compared to less than 10 % for 18:3n-3. This suggests that feeding oils rich in 18:4n-3 could be a superior alternative to feeding oils rich in 18:3n-3 to salmonid species. However, studies examining this strategy have reported mixed results. Miller *et al.* (2007a, freshwater Atlantic salmon) and Miller *et al.* (2008, saltwater Atlantic salmon) found no significant differences in muscle lipid when Atlantic salmon

were fed echium oil, a diet rich in 18:4n-3. When Atlantic salmon (saltwater) were fed SDA-rich soybean (*Glycine* max) oil (Nanton *et al.,* 2012) there was a negative effect on growth and no significant increases in fillet n-3 LC-PUFA when compared to those fed rapeseed oil, however increased levels of 18:4n-3 and 20:4n-3 (eicosatetraenoic acid, ETA) were seen when fed 50 % 18:4n-3 soy oil versus 50 % rapeseed oil. This suggests that 18:4n-3 and 20:4n-3 are more effective precursors for 20:5n-3 than 18:3n-3. In agreement, Cleveland *et al.* (2011), reported even though bioconversion of 18:4n-3 was more efficient than 18:3n-3, a head to head comparison of dietary lipid sources showed no significant differences within the LO fed fish compared to the fish fed other diets. In contrast, Bharadwaj *et al.* (2009) found that when hybrid striped bass were fed 18:4n-3 compared to 18:3n-3 there was a significant increase in muscle 22:6n-3. Codabaccus *et al.* (2011a) reported Atlantic salmon fed echium oil had higher whole-carcass 18:4n-3 and 20:4n-3 in freshwater. In addition, prolonged feeding on the echium oil diet in seawater resulted in higher 18:4n-3, 20:4n-3, 20:5n-3 and docosapentaenoic acid (DPA; 22:5n-3) compared with rapeseed oil fed fish. Furthermore, in an extended feeding trial, Codabaccus *et al.* (2011b) found that providing dietary 18:4n-3 enriched oil to Atlantic salmon promoted increased n-3 LC-PUFA in liver and white muscle in both freshwater and saltwater environments compared to rapeseed oil fed fish. Furthermore, it was identified that increased supply of the n-3 LC-PUFA was due to the 18:4n-3 precursor in both of the aforementioned tissues. It should also be mentioned that the increased n-3 LC-PUFA biosynthesis in fish fed echium oil was not enough to reach comparable n-3 LC-PUFA levels found in white muscle of FO fish.

Almost all of the previous studies used echium oil as the source of 18:4n-3. Echium spp is not a commercial crop and thus, the supply of SDA-rich oil is limited. Recently, our

collaborators at the Bioactive Oils Program (Edmonton, Alberta) have created a genetically modified linseed that produces approximately 26 % 18:4n-3 (% of total lipid). This has the potential of making SDA-rich flax oil commercially available. Furthermore, previous studies in our laboratory have shown that feeding coriander oil, containing 18:1n-12, increases the concentration of 20:5n-3 and 22:6n-3in rainbow trout fed LO (see Chapter 3). Thus, we hypothesise that feeding SDA-rich flax with the addition of coriander oil will result in increased production of 20:5n-3 and 22:6n-3 in rainbow trout.

5.3 MATERIALS AND METHOD

5.3.1 *Oil preparation*

SDA-enriched linseed was gifted from Dr. Randall Weselake (Bioactive Oils Program, University of Alberta). The genetically modified SDA-enriched linseed (Dr. Randall Weselake, University of Alberta) and coriander seed (Diefenbaker Seed Processors, Elbow, SK), were cold pressed at the bioprocessing pilot plant (University of Saskatchewan) using a continuous screw Komet oil press (type CA 59 G3; IBG Monforts Oekotec GmbH & Co. KG, Germany) with a 6 mm choke and operating with a screw speed of 70 rpm for flax seed and 87 rpm for coriander seed. To prevent oxidation of the oils, vitamin E (Lutavit® E 50; BASF Corporation, Florham Park, NJ) and butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO) were added to the oils at levels of 5 g kg^{-1} oil and 4 g kg^{-1} oil respectively and N_2 gas was bubbled through the oils during production. Oils were stored under N_2 at 4 °C until used. Human grade menhaden FO and conventional flax oil was obtained from Bioriginal Ltd., Saskatoon, SK.

5.3.2 *Diet composition and preparation*

The diet formulation and lipid compositions are shown in Tables 5.1 and 5.2. All nutrient values are on a digestible basis. Experimental diets containing coriander oil have calculated values for 18:1n-12 due to difficulty separating this FA from its isomers. Diets were formulated to contain 17.6 MJ kg^{-1} digestible energy, 136.8 g kg^{-1} crude fat and 386.4 g kg^{-1} of digestible CP. The diets met or exceeded all other nutrient requirements of rainbow trout (NRC, 2011). The diets used in the feeding experiment were mixed in a Legacy Hobart Floor Mixer (Hobart Corporation, Troy, OH) for 15 minutes and then cold pelleted in a 3 mm 4822 Hobart Food Grinder (Hobart Corporation, Troy, OH). Following extrusion, the diets were dried in a forced air oven (55 °C, 12 hours), chopped and screened to obtain a uniform pellet size, approximately 10 mm in length.

5.3.3 *Fish husbandry*

Fish used in the experiment were maintained in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2005). Triploid rainbow trout with an initial weight of approximately 42 g were purchased from Wild West Steelhead (Lucky Lake, SK) and maintained in 360 L tanks that were part of a semi-closed recirculation system. Water temperature was maintained at 15 ± 1.5 °C and dissolved oxygen, pH and water temperature were observed and recorded daily. Chlorine, nitrate, nitrite and ammonia were monitored on a weekly basis. Photoperiod was maintained at 14 h light/10 hour dark cycle. The experiment used eighteen tanks of twenty rainbow trout (3 tanks per treatment; 20 fish per tank). Fish were fed twice daily to apparent satiation for 75 days. Before the trial began five fish were selected at random from the whole population, killed by overdose of MS-222 and stored at -80 °C for

analysis to determine initial whole-body fatty acid (WBFA) composition. At the end of the experiment, 3 fish per tank were killed by overdose of MS-222, pooled and stored at -80 °C until analysis.

5.3.1 *Analytical methods*

Experimental diets were ground using a ZM 100 Retsch Mill (Retsch GmbH, Haan, Germany) with a 1 mm screen. Analysis of all samples were conducted in duplicate using the following methods: moisture (AOAC, 1990; method no. 934.01), energy (oxygen bomb calorimetry; Parr Adiabatic Calorimeter, Model 1200), ash (AOAC, 1990; method no. 924.05), gross energy was determined using a 1281 Bomb Calorimeter (Parr Adiabatic Calorimeter, Model 1200) and protein was determined using a Leco protein N^{-1} analyser (Model FP-528, Leco Corporation, St. Joseph, MI). The nitrogen content of samples was obtained using a combustion nitrogen analyser (Leco FP-528, AOAC 1995, method no. 990.03). Crude protein (CP) was estimated by multiplying nitrogen content by 6.25. Whole fish were uniformly ground using a food processor (Moulinex DPA2, France) and stored at -80 °C. Total lipid and FA were determined using the method of Folch *et al.*, (1957). After lipids were extracted, diet and fish samples were sent to SunWest Food Laboratory Ltd. (Saskatoon, SK) for determination of FA composition (MTFAT-01) using gas chromatography (Agilent Technologies, Mississauga, ON). Coriander oil was sent to Lipid Analytical Laboratories analysed (Guelph, ON) for determination of FA composition (Bligh and Dyer, 1959) using gas chromatography (Agilent Technologies, Mississauga, ON).

Table 5.1 Formulations and chemical composition (g kg^{-1}) of the diets

	FO	FO^{+C}	LO	LO^{+C}	SDA	SDA^{+C}
Formulation						
Vegetable oil	120.4	120.4	120.4	120.4	120.4	120.4
Coriander oil	----	15.0	----	15.0	----	15.0
Blood meal	32.0	32.0	32.0	32.0	32.0	32.0
Poultry by-product meal	100.0	100.0	100.0	100.0	100.0	100.0
Dicalcium phosphate	37.5	37.495	37.5	37.495	37.5	37.495
DL-methionine	2.4	2.4	2.4	2.4	2.4	2.4
Pea protein concentrate	243.1	243.1	243.1	243.1	243.1	243.1
Canola protein concentrate	250.0	250.0	250.0	250.0	250.0	250.0
Corn gluten meal	150.0	149.99	150.0	149.99	150.0	149.99
Choline Chloride	4.0	4.0	4.0	4.0	4.0	4.0
Celite	50.0	50.0	50.0	50.0	50.0	50.0
Vitamin mineral premix[1]	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5
Inert marker, Yb_2O_3	0.1	0.1	0.1	0.1	0.1	0.1
Chemical composition						
Dry matter	972.7	966.1	972.5	957.1	968.3	968.3
Crude protein	473.1	474.8	476.9	477.0	473.7	469.7
Gross energy (MJ kg^{-1})	21.8	22.1	22.0	22.0	21.7	21.7
Crude fat	208.3	198.6	207.4	200.3	198.7	232.5
Ash	135.2	131.2	136.3	138.0	139.8	134.7

Fish oil, FO; fish oil + coriander, FO^{+C}; linseed oil, LO; linseed oil + coriander, LO^{+C}; SDA rich LO, SDA; SDA rich LO + coriander, SDA^{+C}; [1]As reported previously (Randall and Drew, 2010)

Table 5.2 Lipid composition (% total fat) of coriander oil and experiment diets

Fatty acid	Coriander oil	FO	FO^{+C}	LO	LO^{+C}	SDA	SDA^{+C}	Reanalysis of SDA^{+C}
14:0	0.16	6.21	5.58	0.13	0.18	0.16	0.61	0.43
15:0	0.04	0.38	0.35	0.07	0.03	0.03	0.14	0.14
16:0	3.58	17.09	15.91	7.62	7.67	8.17	18.62	21.52
18:0	1.06	4.77	4.43	3.83	3.62	3.95	6.91	9.10
20:0	0.10	0.23	0.24	----	0.03	0.03	0.47	3.61
22:0	----	0.05	0.03	0.07	0.03	0.06	0.14	0.58
24:0	----	0.06	0.07	0.10	0.10	0.10	0.27	0.42
Σ SAT[1]	4.93	30.07	27.56	12.01	12.08	13.05	27.55	35.80
14:1n-5	----	0.34	0.22	----	0.03	0.03	0.07	----
15:1n-5	----	0.15	0.12	----	0.03	0.06	0.20	----
16:1n-7	0.14	7.54	6.87	0.79	0.92	0.80	2.17	1.66
17:1n-7	----	1.15	1.04	0.07	0.07	0.03	0.14	----
18:1n-12	69.97	----	1.05[2]	----	1.05[2]	----	1.05[2]	9.67
18:1n-9	8.55	19.35	20.07	26.59	28.03	18.76	39.19	36.58
18:1n-7	0.90	3.16	2.99	1.32	1.37	1.27	2.58	2.40
20:1n-9	0.20	0.31	0.27	----	0.02	0.35	0.14	0.83[5]
22:1n-9	----	0.09	0.10	----	----	0.03	----	0.29[6]
24:1n-9	----	0.28	0.25	0.07	0.03	0.03	1.56	0.14
Σ MUFA[3]	79.76	33.33	36.84	29.13	33.37	21.42	60.02	54.71
18:2n-6	15.04	7.81	8.83	17.68	17.53	9.31	6.50	6.96
18:3n-6	0.13	0.37	0.20	0.16	0.10	10.33	0.27	0.21
20:2n-6	----	0.12	0.14	0.04	0.03	0.03	----	0.26
20:3n-6	----	0.12	0.10	----	0.03	0.46	0.07	----
20:4n-6	----	0.76	0.71	0.07	0.03	0.03	----	0.10
22:2n-6	----	0.08	0.07	----	0.02	----	0.07	----
22:4n-6	----	0.64	0.58	----	0.03	0.03	0.55	----
22:5n-6	----	0.25	0.25	----	0.03	----	0.41	0.02
Σ n-6	15.17	10.13	10.86	17.94	17.81	20.18	7.87	7.55
18:3n-3	0.13	2.70	2.45	40.56	36.02	24.54	2.03	1.64
18:4n-3	----	2.18	1.79	----	0.15	19.48	0.33	0.30
20:3n-3	----	0.15	0.15	0.16	0.15	0.16	0.34	----
20:4n-3	----	0.02	0.03	----	----	----	----	----
20:5n-3	----	11.84	11.19	0.07	0.13	0.03	0.34	----
21:5n-3	----	0.50	0.48	----	0.02	0.49	0.33	----
22:5n-3	----	1.49	1.36	0.07	0.03	0.02	0.14	----
22:6n-3	----	7.09	6.71	----	0.08	0.02	0.14	----
Σ n-3	0.13	25.95	24.14	40.84	36.58	44.72	3.63	1.94
Σ PUFA[4]	15.30	36.56	35.45	58.87	54.56	65.03	12.44	9.50
Others	----	0.05	0.17	----	----	0.51	----	----

See Table 5.1 for abbreviations; [1]Includes 10:0, 12:0, 13:0, 17:0, 19:0, and 23:0; [2]Calcultaed values; [3]Includes 18:1n-12; [4]Includes 16:2, 16:3, 16:4, 18:2; ---- = not detected; [5]Includes 20:1n-11, 22:1n-11; [6]Includes 22:1n-11

5.3.2 *Statistical analysis*

The experiment was set up as a completely randomised design with a 3 x 2 factorial arrangement of treatments: 3 oils (fish, conventional flax, and SDA-enriched flax) and 2 levels of coriander oil (0 and 15 g kg^{-1}). The fish oil diets were used only as reference diets and were not included in the statistical analysis. Data were analysed using a 2 x 2 factorial design, however interactions between the two factors were significant ($P < 0.05$) and therefore the data were re-analysed as a completely randomised design with 4 treatments using the General Linear Model of PASW (IBM, Chicago, USA, V. 19.0, 2011). Data are expressed as means ± S.E.M. (3 fish pooled per tank, 3 tanks per treatment; $N = 3$). When significant differences between treatments were observed, means were separated using the Ryan–Einot–Gabriel–Welsch-F test. Treatment effects were considered significant when $P < 0.05$.

5.4 RESULTS

The FA composition of the SDA diet differed markedly from SDA^{+C} diet. Total saturates went from 13.05 % to 27.55 %, monounsaturates from 21.42 % to 60.02 %, n-6 PUFA from 20.18 % to 7.87 % and n-3 PUFA from 44.72 % to 3.63 % in the SDA and SDA^{+C} diets respectively (Table 5.2). Similar changes were observed in total MUFA and n-3 PUFA when coriander oil was added to the LO diet but the differences observed were smaller. To confirm these unexpected differences, the SDA^{+C} diet was reanalysed in another laboratory (Lipid Analytical Laboratories, Guelph, ON) and similar results were obtained, except for 18:1n-12 (Table 5.2). In the original analysis, 18:1n-12 was (1.05%). During reanalysis by the University of Guelph, a value of 9.67 % was determined for 18:1n-12. Coriander oil contained 69.97 % 18:1n-12, as well as high levels of 18:1n-9 and 18:2n-6 (Table 5.2). No significant differences were found for ADG, SGR, ADFI, FCR or mortality between treatments ($P < 0.05$; Table 5.3).

The WBFA content of fish at the beginning of the experiment and the end of the experiment is shown in Table 5.4. Total SFA and 14:0, 15:0 and 16:0 were significantly higher in the SDA^{+C}-fed fish than fish fed the other 3 diets ($P < 0.05$). The concentration of 22:0 was significantly higher in fish fed either of the LO diets compared to those fed either of the SDA diets. The concentration of 24:0 was significantly higher in the fish fed the SDA^{+C} diet compared to those fed the LO diet while the LO^{+C} and the SDA diet were intermediate to the 2 diets.

The concentration of total MUFA were significantly higher in the LO^{+C} and the SDA^{+C} than in the SDA diet while the LO diet was not significantly different from either group ($P < 0.05$). The level of 18:1n-9 was significantly higher in the fish fed the LO diet compared to fish fed the SDA diet with the other 2 treatment groups not significantly different from either treatment ($P < 0.05$).

The concentrations of n-6 PUFA were not significantly different between treatment groups. However, 18:3n-6, 20:3n-6 and 20:4n-6 concentrations were significantly higher in fish fed the SDA diets compared to those fed the LO diets. In contrast, the concentrations of 20:2n-6 and 22:2n-6 were higher in the fish fed the LO diets than those fed the SDA diets.

Total n-3 PUFA were significantly higher in the fish fed the SDA diet than those fed the SDA^{+C} diet while the fish fed the LO diets were intermediate ($P < 0.05$). The concentration of 18:3n-3 was significantly higher in fish fed the LO diets compared to those fed the SDA diet while the SDA^{+C} fed fish had significantly lower concentrations than the other 3 groups. For 18:4n-3 fish fed the LO diets had significantly lower tissue concentrations than those fed the SDA^{+C} and fish fed the SDA diet had significantly higher 18:4n-3 concentrations than the other 3 groups ($P < 0.05$). The concentrations of 20:5n-3 and 22:6n-3 were significantly higher in the fish fed the SDA^{+C} diet than those fed the other 3 diets ($P < 0.05$).

Table 5.3 Growth parameters of fish fed diets containing fish, canola, flax or camelina oils with and without coriander oil

Growth Parameters	FO	FO^{+C}	LO	LO^{+C}	SDA	SDA^{+C}
Initial weight (g)	41.5 ± 2.1	53.7 ± 3.3	42.2 ± 2.7	42.3 ± 2.1	44.7 ± 1.7	42.0 ± 2.9
Final weight (g)	84.0 ± 6.5	75.8 ± 1.2	78.7 ± 12.7	83.1 ± 11.3	76.8 ± 2.3	61.6 ± 3.3
Average daily gain[1]	0.6 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
Specific growth rate[2] (% d^{-1})	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.0
Average daily feed intake[3] (g fish d^{-1})	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.9	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.3
Feed conversion ratio[4] (g feed g $gain^{-1}$)	1.0 ± 0.1	2.0 ± 0.1	1.2 ± 0.3	1.4 ± 0.1	1.5 ± 0.3	1.7 ± 0.2
Survival[5]	90.0 ± 1.7	88.3 ± 2.4	88.3 ± 1.6	86.7 ± 1.6	85.0 ± 2.0	85.0 ± 0.1

Values are given as a percentage (mean ± S.E.M.). Values within the same row with different superscripts are significantly different. $P = 0.05$. n = 3

[1]Average daily gain = final weight - initial weight / number of days on trial

[2]Specific growth rate = ([ln final weight – ln initial weight]) / time (days) x 100)

[3]Average daily feed intake = total daily feed intake / number of days on trial

[4]Feed conversion ratio = total feed intake / average daily gain

[5]Survival – survival during experiment

Table 5.4 Whole body fatty acid (WBFA) composition (% total fat) of rainbow trout fed FO, LO and SDA diets with and without coriander oil for 75 d

Fatty Acid	Initial Fish	FO	FO^{+C}	LO	LO^{+C}	SDA	SDA^{+C}
12:0	0.13 ± 0.01	0.14 ± 0.03	0.10 ± 0.00	0.09 ± 0.01	0.11 ± 0.02	0.09 ± 0.02	0.10 ± 0.01
14:0	5.17 ± 0.06	5.29 ± 0.13	4.69 ± 0.10	2.10 ± 0.02^{a}	2.10 ± 0.28^{a}	2.30 ± 0.09^{a}	3.00 ± 0.12^{b}
15:0	0.39 ± 0.00	0.37 ± 0.01	0.34 ± 0.01	0.18 ± 0.00^{a}	0.20 ± 0.02^{a}	0.20 ± 0.01^{a}	0.26 ± 0.01^{b}
16:0	18.92 ± 0.02	17.72 ± 0.40	16.40 ± 0.17	12.61 ± 0.51^{a}	12.53 ± 0.68^{a}	13.06 ± 0.49^{a}	15.38 ± 0.48^{b}
18:0	4.49 ± 0.09	4.43 ± 0.08	4.14 ± 0.05	4.06 ± 0.23	3.85 ± 0.13	4.15 ± 0.21	4.46 ± 0.15
20:0	0.14 ± 0.01	0.21 ± 0.07	0.20 ± 0.07	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.17 ± 0.04
22:0	0.11 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.61 ± 0.04^{b}	0.55 ± 0.02^{b}	0.23 ± 0.01^{a}	0.16 ± 0.01^{a}
23:0	0.06 ± 0.01	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.03	0.05 ± 0.00
24:0	0.09 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	0.06 ± 0.00^{a}	0.07 ± 0.00^{ab}	0.07 ± 0.00^{ab}	0.08 ± 0.00^{b}
Σ SAT[1]	30.73 ± 0.37	29.12 ± 0.77	26.78 ± 0.36	20.30 ± 0.85^{a}	20.11 ± 1.19^{a}	20.91 ± 0.70^{a}	24.40 ± 0.76^{b}
14:1n-5	0.42 ± 0.24	0.24 ± 0.04	0.21 ± 0.04	0.11 ± 0.00	0.09 ± 0.04	0.10 ± 0.03	0.15 ± 0.01

16:1n-7	7.54 ± 0.06	7.63 ± 0.24	7.07 ± 0.13	3.67 ± 0.13^{a}	3.81 ± 0.49^{a}	3.96 ± 0.15^{a}	5.08 ± 0.23^{b}
17:1n-7	0.80 ± 0.01	0.92 ± 0.01	0.82 ± 0.02	0.36 ± 0.01^{a}	0.36 ± 0.04^{a}	0.43 ± 0.02^{ab}	0.49 ± 0.03^{b}
18:1n-12	-----	-----	1.95 ± 0.44	-----	2.34 ± 0.20	-----	2.37 ± 0.04
18:1n-9	17.40 ± 0.01	20.51 ± 0.33	20.61 ± 0.66	23.68 ± 1.40^{b}	23.18 ± 0.94^{ab}	19.35 ± 0.80^{a}	21.07 ± 0.42^{ab}
18:1n-7	3.35 ± 0.00	3.62 ± 0.04	3.41 ± 0.04	2.22 ± 0.08^{a}	2.24 ± 0.20^{a}	2.30 ± 0.04^{ab}	2.71 ± 0.07^{b}
20:1n-11	1.35 ± 0.00	0.77 ± 0.39	0.80 ± 0.40	0.68 ± 0.30	0.77 ± 0.35	0.60 ± 0.26	1.05 ± 0.05
20:1n-9	0.22 ± 0.00	0.67 ± 0.40	0.71 ± 0.45	0.12 ± 0.01	0.43 ± 0.30	0.42 ± 0.31	0.16 ± 0.01
22:1n-11	0.57 ± 0.06	0.32 ± 0.02	0.36 ± 0.02	0.22 ± 0.00	0.24 ± 0.05	0.25 ± 0.01	0.34 ± 0.04
22:1n-9	0.20 ± 0.00	0.16 ± 0.01	0.17 ± 0.00	0.16 ± 0.04	0.21 ± 0.02	0.13 ± 0.01	0.15 ± 0.01
24:1n-9	0.38 ± 0.00	0.27 ± 0.01	0.27 ± 0.01	0.18 ± 0.01^{a}	0.20 ± 0.02^{a}	0.20 ± 0.01^{a}	0.27 ± 0.01^{b}
Σ MUFA[2]	32.27 ± 0.12	35.13 ± 0.63	36.40 ± 0.44	31.40 ± 1.94^{ab}	33.86 ± 1.71^{b}	27.72 ± 0.76^{a}	33.86 ± 0.78^{b}
18:2n-6	7.50 ± 0.01	6.29 ± 1.18	7.78 ± 1.18	12.37 ± 4.26	14.21 ± 4.29	9.91 ± 2.87	7.66 ± 1.29
18:3n-6	0.15 ± 0.00	0.22 ± 0.02	0.19 ± 0.01	0.40 ± 0.01^{a}	0.34 ± 0.03^{a}	4.19 ± 0.33^{c}	2.31 ± 0.20^{b}
20:2n-6	0.45 ± 0.00	0.49 ± 0.02	0.55 ± 0.00	0.55 ± 0.03^{b}	0.58 ± 0.04^{b}	0.33 ± 0.01^{a}	0.38 ± 0.02^{a}
20:3n-6	0.29 ± 0.02	0.27 ± 0.00	0.28 ± 0.0	0.35 ± 0.02^{a}	0.35 ± 0.02^{a}	1.18 ± 0.07^{c}	0.84 ± 0.03^{b}
20:4n-6	1.14 ± 0.00	0.91 ± 0.02	0.94 ± 0.03	0.59 ± 0.04^{a}	0.60 ± 0.07^{a}	0.81 ± 0.03^{b}	1.02 ± 0.05^{c}
22:2n-6	0.07 ± 0.01	0.03 ± 0.00	0.05 ± 0.03	0.06 ± 0.00^{b}	0.07 ± 0.00^{b}	0.05 ± 0.01^{ab}	0.03 ± 0.00^{a}
22:4n-6	0.66 ± 0.0	0.64 ± 0.02	0.63 ± 0.01	0.29 ± 0.00^{a}	0.29 ± 0.05^{a}	0.34 ± 0.01^{a}	0.45 ± 0.02^{b}
22:5n-6	0.31 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.18 ± 0.01^{a}	0.20 ± 0.01^{a}	0.19 ± 0.02^{a}	0.27 ± 0.02^{b}
Σ n-6	10.56 ± 0.02	9.09 ± 1.12	10.62 ± 1.10	14.78 ± 4.20	16.63 ± 4.08	16.99 ± 2.49	12.96 ± 1.40
18:3n-3	1.25 ± 0.01	1.93 ± 0.09	1.73 ± 0.04	16.94 ± 1.45^{c}	14.16 ± 0.65^{c}	9.86 ± 0.75^{b}	5.68 ± 0.33^{a}
18:4n-3	1.00 ± 0.00	1.46 ± 0.18	1.59 ± 0.12	3.08 ± 0.11^{a}	2.40 ± 0.13^{a}	9.63 ± 0.68^{c}	5.14 ± 0.45^{b}
20:3n-3	0.09 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.12 ± 0.04	0.08 ± 0.01	0.11 ± 0.02	0.07 ± 0.00
20:4n-3	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.08 ± 0.02	0.05 ± 0.00
20:5n-3	6.88 ± 0.00	6.82 ± 0.08	6.59 ± 0.17	3.30 ± 0.01^{a}	3.05 ± 0.31^{a}	3.75 ± 0.05^{ab}	4.38 ± 0.15^{b}
21:5n-3	0.76 ± 0.00	0.72 ± 0.01	0.67 ± 0.02	0.95 ± 0.05^{a}	0.84 ± 0.07^{a}	1.52 ± 0.07^{b}	1.06 ± 0.02^{a}
22:5n-3	2.43 ± 0.01	2.23 ± 0.04	2.31 ± 0.01	1.22 ± 0.01^{a}	1.22 ± 0.19^{a}	1.38 ± 0.02^{a}	1.79 ± 0.05^{b}
22:6n-3	12.71 ± 0.02	11.61 ± 0.45	11.80 ± 0.29	6.86 ± 0.27^{a}	7.03 ± 0.92^{a}	7.27 ± 0.14^{a}	9.74 ± 0.46^{b}
Σ n-3	25.15 ± 0.00	24.89 ± 0.39	24.81 ± 0.51	32.50 ± 1.82^{ab}	28.82 ± 1.18^{ab}	33.59 ± 1.29^{b}	27.91 ± 0.27^{a}
Σ PUFA	36.52 ± 0.05	34.70 ± 1.35	36.09 ± 0.64	47.64 ± 2.45	45.83 ± 2.91	50.93 ± 1.42	41.38 ± 1.54
Others	0.92 ± 0.06	1.03 ± 0.09	0.74 ± 0.05	0.31 ± 0.03^{ab}	0.20 ± 0.05^{a}	0.44 ± 0.05^{b}	0.37 ± 0.04^{ab}

Values are means ± S.E.M.; n = 3. [1]Includes: 10:0, 13:0, 17:0, 19:0, and 23:0; [2]Includes: 15:1n-5; [abc]Means in a common row without a common letter differ; $P = 0.05$. The initial sample was not included in the statistical analysis. ---- = Not detected

5.5 DISCUSSION

The FA profile of coriander oil in this experiment agrees with the literature, containing mainly 16:0, 18:1n-12, 18:1n-9 and 18:2n-6 (Weber *et al*., 1995; Reiter *et al*., 1998; Sriti *et al.,* 2010). Coriander oil also contains biologically active compounds including alcohols, linalool and geraniol, sterols, tocopherols, hydrocarbons, ketones, coumarins and furanocoumarins (Ramadan *et al.,* 2008; Burdock *et al.,* 2009). In previous studies, coriander oil was refined, bleached and deodorised (Weber *et al.,* 1995, 1997, 1999; Richter *et al.,* 1996; Ramadan *et al.,* 2008). However, in the present study, coriander oil was fed as is. Despite this, the growth and feed intake of the fish were not affected. Despite this, the strong odour of coriander oil would likely produce a product with unacceptable sensory qualities and the use of coriander oil will require that it be deodorised before use. This may explain the increase in FCR when fish were fed FO containing coriander oil (FO^{+C}), however further research is needed to verify this as a signifncat difference was not found when compared to the other treatments.

When coriander oil was added to the LO and SDA diets it resulted in a marked apparent decrease in 18:3n-3 and 18:2n-6. In a previous trial performed in our lab the same pattern was seen when coriander oil was added to VO-based diets (See Chapter 4). To ensure accuracy, the SDA^{+C} diet was re-analysed in a different laboratory and the FA composition of the diets was the same. This supports the possibility of an interaction between 18:1n-12 and other 18 carbon FA during analysis, as this decrease is not seen in the diets not containing coriander oil. In support of this, the FA composition of the fish fed the coriander diets had concentrations of 18:3n-3 and 18:2n-6 that were greater than would be predicted based on the concentrations of these FA in the diets. Nevertheless, further research needs to be performed to verify that an interaction between

coriander oil and 18:3n-3 and 18:2n-6 exists and to develop methods to measure these FA accurately.

The rate-limiting step in the conversion of 18:3n-3 to LC-PUFA is Δ6 desaturase (in humans: Sprecher, 1981; in fish: Tocher *et al.,* 2006). This bottleneck can be bypassed through the use of 18:4n-3-rich oils in aquaculture feeds. Previous studies have reported that the replacement of 18:3n-3-rich VO with 18:4n-3-rich VO increased muscle concentrations of 22:6n-3 in hybrid striped bass (Bharadwaj *et al.,* 2010) and increased whole-carcass 18:4n-3 and 20:4n-3 in freshwater Atlantic salmon and prolonged feeding on the 18:4n-3-rich oil in seawater resulted in higher 18:4n-3, 20:4n-3, 20:5n-3 and docosapentaenoic acid (DPA; 22:5n-3) compared with fish fed rapeseed oil (Codabaccus *et al.,* 2011). However, Cleveland *et al.,* (2011) and Miller *et al.,* (2007a, 2008) reported no significant increase in tissue 22:6n-3 when 18:4n-3-rich echium oil was fed to rainbow trout or Atlantic salmon. The current study found no significant increases in 20:5n-3 or 22:6n-3 in fish fed the SDA diet compared to those fed the LO diets. This suggests that Δ6 desaturase activity was not limiting in the bioconversion of 18:3n-3 to LC-PUFA in rainbow trout and thus, the presence of 18:4n-3 in the SDA diet did not significantly increase the rate of biosynthesis of 20:5n-3 or 22:6n-3.

It has been previously reported that high dietary and tissue concentrations of 20:5n-3 and 22:6n-3 decrease the activity of the first Δ6 desaturase step in the n-3 FA pathway, through negative feedback (Mozaffarian *et al*., 2005). Thus, the presence of feed ingredients, such as FM and FO, which contain 20:5n-3 and 22:6n-3, may reduce the desaturation of 18:3n-3 by lowering Δ6 desaturase activity (Cho *et al.,* 1999; Nakamura and Nara, 2004; Mozaffarian *et al*., 2005). In this case, feeding 18:4n-3 would be expected to increase the biosynthesis of 20:5n-3 and 22:6n-3 because it would bypass this step. The diets used in the current study contained no 20:5n-3 and

22:6n-3 and thus, negative feedback on FA desaturation due to dietary FA composition would not have occurred. In this case, the Δ6 desaturase activity of fish fed the LO diets may not have limited the production of LC-PUFA from 18:3n-3 compared to 18:4n-3. In support of this, studies where significant amounts of dietary 20:5n-3 and 22:6n-3 (Bharadwaj *et al.,* 2010; Codabaccus *et al.,* 2011) were present reported that fish fed 18:4n-3 had significantly higher tissue levels of 20:5n-3 and 22:6n-3 or 22:5n-3, respectively. In contrast, experiments that fed diets devoid of 20:5n-3 and 22:6n-3 (Cleveland *et al.,* 2011) reported no significant differences in tissue concentrations of 20:5n-3 and 22:6n-3 when fish were fed 18:4n-3 compared to 18:3n-3. This suggests that feeding 18:4n-3 is only of significant benefit if diets also contain LC-PUFA, 20:5n-3 and 22:6n-3.

It is believed that 18:1n-12, found in coriander oil, has the ability to inhibit the desaturation of LA (n-6 FA pathway) in rats by pseudo-product mediated inhibition or in other words, acting as 18:3n-6, a product of Δ6 desaturase (Weber *et al.,* 1995, 1997, 1999). This is supported by the results reported in Chapter 3, where the production of 20:4n-6 was numerically decreased while the tissue concentrations of 22:6n-3 were significantly increased. This supports the hypothesis that 18:1n-12 affects the activity of Δ6 desaturase. In the present experiment, there was an interaction between coriander oil and the two flax oils on the levels of 22:6n-3 in fish tissues. Fish fed the coriander free diets and the LO diet with coriander oil (LO^{+C}) had no significant differences in tissue 22:6n-3 concentrations. However, when the trout were fed a diet high in 18:4n-3 with coriander (SDA^{+C}), there was a significant increase in 22:6n-3. In contrast, when coriander oil was added to VO-based diets in Chapter 3, it caused a significant increase in 22:6n-3. The major difference between the present experiment and the previous one was the length of time the fish were fed the experimental diets. In Chapter 3, fish were fed for 112 days

while in the present experiment they were fed for only 75 days. Thus, while the conversion of 18:3n-3 to 18:4n-3 may have been increased, the 75-day feeding period may not have been sufficient to allow the accumulation of significantly more 22:6n-3 in the fish tissue in fish fed the LO^{+C} diet. The significant increase in the tissue concentrations of 22:6n-3 in the fish fed the SDA^{+C} diet may have been due to the additive effects of increased Δ6 desaturase conversion of 18:3n-3 to 18:4n-3 plus the higher bioefficacy of the 18:4n-3 present in the SDA^{+C} diet. Thus, even though neither effect was significant by itself, the combination of the two effects resulted in significant levels of 22:6n-3.

The importance of 20:4n-6 has been largely neglected in aquaculture research due to the focus on 20:5n-3 and 22:6n-3. 20:4n-6 is the precursor to pro-inflammatory eicosanoids, which have a higher inflammatory activity than eicosanoids derived from 20:5n-3. Furthermore, 20:4n-6 is the preferred substrate for eicosanoid production in fish (Tocher *et al.,* 1996). In addition, 20:5n-3 and its eicosanoids competitively inhibit the formation and action of 20:4n-6 derived eicosanoids, respectively (Sargent *et al*., 1999). If the balance between 20:4n-6 and 20:5n-3 FA is not maintained it can hinder immune response. Sargent *et al*. (1999) reported that increased tissue ratios of 20:4n-6 to 20:5n-3 result in greater inflammatory responses compared to low tissue ratios of 20:4n-6 to 20:5n-3. Although the optimum ratio of 20:4n-6 to 20:5n-3 and tissue concentrations of 20:4n-6 are unknown in fish (NRC, 2011) it is possible that the requirement for 20:4n-6, although probably very small, may be overlooked causing increased stress in fish. This is mainly the case when VO are used as replacements for FO and FM in aquaculture rations as VO do not contain 20:4n-6 (Bell and Sargent, 2003). The feeding of 18:1n-12 might further reduce 20:4n-6 concentrations and negatively affect inflammatory responses in salmonids.

The SDA-enriched LO was also rich in 18:3n-6; the desaturation product of 18:2n-6.

Thus, the Δ6 desaturase bottleneck for the first desaturation step in n-6 FA biosynthesis would be bypassed for n-6 FA as well. In support of this, feeding the SDA-enriched diet resulted in a significant increase in tissue levels of 20:4n-6. Furthermore, fish fed the SDA^{+C} diet had a further significant increase in tissue levels of 20:4n-6. Given the hypothesis that 18:1n-12 decreases the conversion of 18:2n-6 by Δ6 desaturase, this result is difficult to explain. It suggests that 18:1n-12 might have an effect on subsequent steps in the biosynthetic pathway for LC-PUFA. It has been reported that increased levels of 20:4n-6 during smoltification improves immune responses and reduces stress. Furthermore, Bell and Sargent (2003) reported that larval gilthead sea bream had improved growth, survival rates and resistance to handling stress when 20:4n-6 was increased in the diet, suggesting fish may benefit from 20:4n-6 supplementation. They noted that even when excess 18:2n-6 is supplied in the diet, 20:4n-6 production can still be limited due to enzyme inhibition and preference. Thus, the use of 18:3n-6 rich LO combined with 18:1n-12 might increase the concentration of 20:4n-6 in fish tissues and provide a better balance of n-6 and n-3 eicosanoids than conventional flax oil.

Even though these results show increases in 22:6n-3 when coriander oil is present, feeding VO to rainbow trout does not result in 22:6n-3 levels similar to fish fed FO. However, it does suggest that addition of positional isomers of 18:1n-9, such as 18:1n-12, found in coriander oil to VO may be a useful approach to increasing LC-PUFA in aquaculture fish. Further research might provide insight into the mechanism of action of 18:1n-12 and allow further improvements in the bioconversion of 18:3n-3 to LC-PUFA.

6 GENERAL DISCUSSIONS

The future of the aquaculture industry is not sustainable if alternative feed ingredients are not found to reduce the industry's reliance on FM and FO. Plant based lipid and protein sources are currently the most readily available, cost effective products with low levels of organic contaminants. However, the major limitation of FM and FO replacement is the significant reduction of health-promoting n-3 LC-PUFA, 20:5n-3 and 22:6n-3, due to alterations in FA metabolism as VO lack these LC-PUFA. Even though it is well established that freshwater salmonids have the ability to convert n-6 and n-3 FA precursors to 20:5n-3 and 22:6n-3, the low efficiency of the LC-PUFA biosynthetic pathway limits manipulation through dietary modification.

While supplies of FO and FM are static, many studies still provide minimal levels of these ingredients in the diet either throughout the full duration of the experiment or during a washout period to ensure optimal fish growth and to boost tissue levels of 20:5n-3 and 22:6n-3 prior to harvest. However, it has been shown that providing a source of these LC-PUFA in the diet can cause negative feedback on fatty acyl desaturases within the n-3 FA pathway. The exact mechanism and level this occurs at has yet to be determined. In the present studies, diets were formulated to contain no FM and/or FO in order to remove potential feedback. However, it was found that providing some level of 20:5n-3 and 22:6n-3 in the diet may be beneficial when feeding 18:4n-3-rich diets, bypassing the first Δ6 desaturase, compared to feeding diets rich in 18:3n-3 as it resulted in increased 22:6n-3. In future studies, a comparison of SDA diets with and without 20:5n-3 and 22:6n-3 would be beneficial in determining if negative feedback has an effect on Δ6 desaturase.

As previously mentioned, feeding conventional VO to fish does not provide acceptable

levels of 20:5n-3 and 22:6n-3 compared to FO fed fish. Therefore, it is essential to find better alternatives through the development of plants producing LC-PUFA or the addition of specalised oil to maintain or enhance the levels of 20:5n-3 and 22:6n-3 in VO-fed fish. It has been demonstrated in rats, that ingestion of 18:1n-12, in coriander oil, manipulates the n-6 FA pathway, causing a reduction in 20:4n-6 in the liver, heart and blood, however the effects on the n-3 FA pathway were never clearly examined. Only a limited number of studies exist which examine the metabolic and biological effects of positional isomers of 18:1n-9, such as 18:1n-12. In general, good separation of long chain FA can prove difficult as higher molecular weight FA tend to have sub-optimal chromatographic properties. Furthermore, analysis of FA isomers can be difficult due to similarities in boiling point. Resolutions are also poor due to low temperature programs, which can lead to elution issues of long chain isomers. In order to fully understand the complexity of the effects coriander oil, specifically 18:1n-12, has on FA metabolism, a method using mass spectrometric detection is needed to clearly separate 18:1n-12 from its positional isomers. This will also aid in the discovery of its mechanism of action.

The present studies demonstrate that freshwater rainbow trout fed VO containing 18:1n-12, have the ability to increase the bioconversion of 18:3n-3 to 20:5n-3 and/or 22:6n-3 in the fillet, liver and, whole carcass and decrease bioconversion of 18:2n-6 to 20:4n-6 in liver and muscle. However, feeding a diet rich in 18:3n-6 caused increased whole body production of 20:4n-6. In addition, the presence of 18:1n-12 in the SDA-enriched diet reduced 18:3n-6, suggesting increased conversion to 20:4n-6. Feeding diets high in 18:3n-6 might be advantageous during smoltification as increased levels of 20:4n-6 during this period has been shown to reduce stress and improve immune function.

Due to the limited amount of research performed using positional isomers of 18:1n-9 it

would be beneficial to perform an *in vitro* assay comparing the addition of 18:1n-12 to radiolabelled n-3 and n-6 FA substrates in individual organ tissues. This would help determine if the effect of 18:1n-12 were tissue dependent, as Weber *et al.* (1999) proposed that 18:1n-12 may inhibit n-3 FA production of DHA in rat brain, which would counteract the ideas found in the present studies. In addition, the questions still remains as to the ideal level of coriander oil, as results from Chapter 4 could not definitively suggest the most optimal levels of inclusion. Additional studies are needed to determine this inclusion level.

In Chapter 4, Δ6 desaturase gene expression was examined to further identify if Δ6 desaturase is the enzyme affected by feeding 18:1n-12 to rainbow trout, as was seen in previous studies in rats. It was found that Δ6 desaturase gene expression decreased with increasing inclusion of coriander oil, however due to the relative nature of this assay, it could not be determined which Δ6 desaturase was being reduced as the same enzyme is utilised within a pathway more than once. In addition, ACO oxidase activity was measured to determine if the rate-limited step in peroxisomal β-oxidation was the cause of increased 22:6n-3, however no significant differences were found. This led to the idea that 18:1n-12 may not affect Δ6 desaturase and probably affects bioconversion between the first Δ6 desaturase and peroxisomal β-oxidation. Therefore in Chapter 5, the first Δ6 desaturase was bypassed by feeding diets rich in 18:4n-3. This caused an increase in bioconversion to 22:6n-3 when 18:1n-12 was present, further suggesting that the effect of 18:1n-12 may not be on the first Δ6 desaturase but another enzyme within the FA pathway. In order to clearly identify the effect of 18:1n-12 on enzyme activity and gene expression, alternative techniques must be investigated such as the WBFA balance methods as proposed by Turchini *et al.* (2007). This technique calculates the activity of each enzyme with the n-6 and n-3 FA pathways separately with regard to its FA precursor and product and may

provide a better understanding of how 18:1n-12 affects FA metabolism in fish species. It would be beneficial to perform an *in vitro* radiolabelled assay whereby [1-^{14}C] 18:1n-12 would be added to each substrate in the n-3 and n-6 FA pathways to determine if elongase 2, elongase 5, Δ5 desaturase or the second Δ6 desaturase is being affected. Furthermore, implementing this technique in myocytes and adipocytes would help identify how 18:1n-12 affects muscle tissue and adipogenesis.

With the global population expected to exceed 9 billion by 2050, the expansion of the available food supply is essential. Not only the quantities must be increased but also the nutritional quality of food must be maintained. Fish are the primary source of LC-PUFA in human diets but as aquaculture becomes the primary source of fish, new sources of LC-PUFA must be found for aquaculture feed production. Although the use of genetically modified plant ingredients has been criticised, it offers a feasible approach to sustainable production of LC-PUFA. A large number of studies have been conducted to assess the effect of feeding genetically modified plant ingredients to animals (reviewed by Sissener *et al.,* 2011). Thus far no study has reported that the feeding of genetically modified plant ingredients have caused detrimental effects on fish health or nutritional quality. This suggests that the use of genetically modified plants for the production of LC-PUFA oils for use in aquaculture is not only feasible but also safe.

Coriander oil was not refined, bleached, and/or deodorised in these experiments as with other experiments, (Weber *et al.,* 1995, 1997, 1999; Ritcher *et al.,* 1996; Ramadan *et al.,* 2008) yet growth and feed intake were not affected. Due to the presence of biologically active compounds in the oil (Ramadan *et al.,* 2008; Burdock *et al.,* 2009) precaution must be taken to ensure these compounds do not cause physiological effects on fish. Therefore, future studies in

fish should refine, bleach, and deodorise the coriander oil to eliminate possible interactions.

The present studies examined trout at the juvenile life stage in a freshwater environment. However, given the complexity of lipid metabolism in fish species, environmental conditions, and life stage, it would be useful to test the effects of coriander oil at different life stages in both warm and cold, freshwater and saltwater environments to determine the most effect way to utilise its benefits.

The sustainability of aquaculture depends on the continuing improvement of production systems, genetics, health, environmental impact and feed resources. Meeting these challenges will increase production of safe and nutritious food for humanity by harnessing the productive capacity of the oceans.

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Appendix 1

Photo by: Kyla Randall, 2009

Figure 7.1 Komet vegetable oil press

Appendix 2

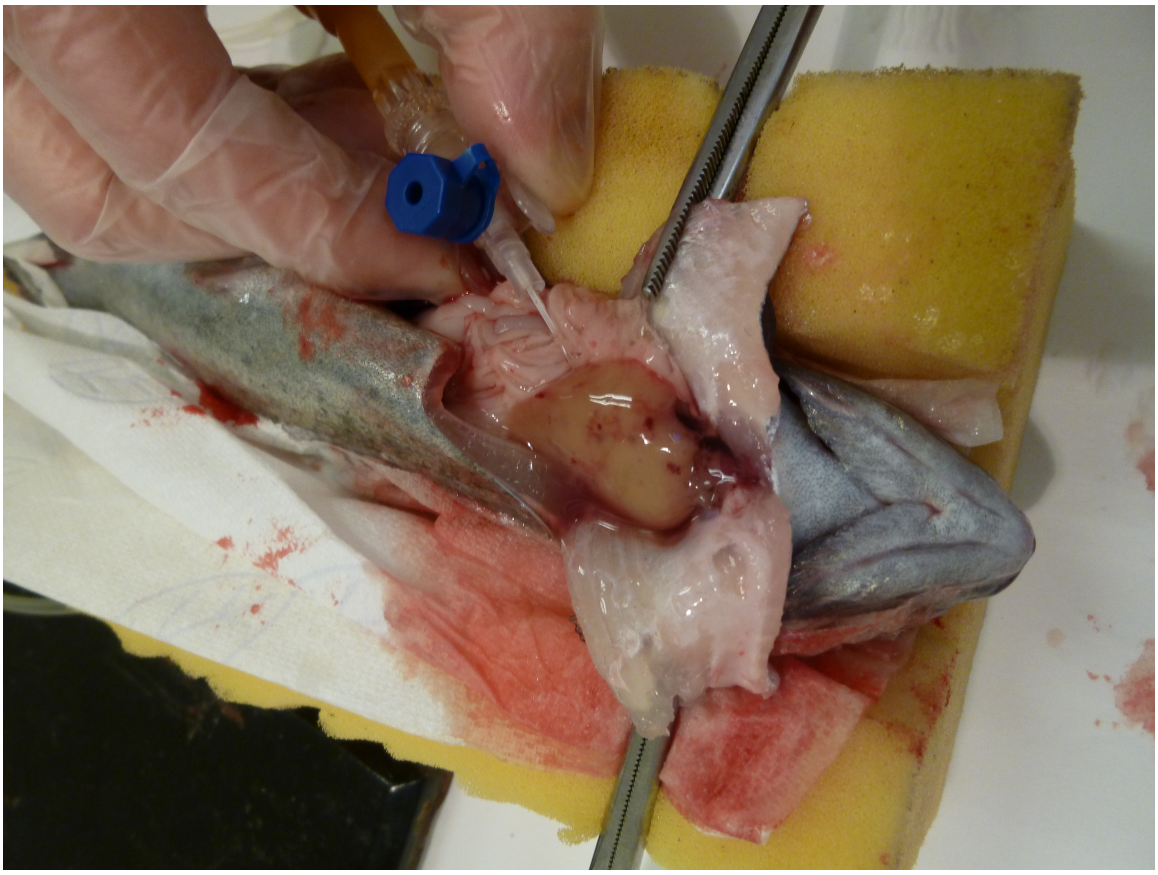

Photo by: Marte Kjær, 2011

Figure 7.2 Liver perfusion

APPENDIX 3

Photo by: Marte Kjær, 2011

Figure 7.3 My first liver perfusion